

THESIS / THÈSE

MASTER IN BIOCHEMISTRY AND MOLECULAR AND CELLULAR BIOLOGY

Effects of corticosteroids on menisci and synovial fluid ? A study in an ovine model

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UNIVERSITY OF NAMUR

Faculty of Sciences

Effects of corticosteroids on menisci and synovial fluid?

A study in an ovine model.

**Master thesis submitted for obtaining
the academic grade of master 120 in biochemistry, and cellular and molecular biology**

Giulia GRISANTI

January 2016

Quels sont les effets des corticostéroïdes sur le ménisque et le liquide synovial? Une étude sur modèle ovin.

GRISANTI Giulia

Résumé

L'ostéoarthrite du genou est une pathologie caractérisée par une dégradation du cartilage articulaire ainsi que par une inflammation de la membrane synoviale qui accélère cette dégradation. Cette maladie touche l'ensemble des composants anatomiques du genou, à savoir le cartilage articulaire, les ménisques, la membrane synoviale, le liquide synovial, les ligaments et les os. Un des traitements couramment utilisé est l'injection intra articulaire de corticostéroïdes. Malgré l'utilisation massive de ces médicaments, leur balance bénéfices-risques est encore peu étudiée. Des études permettant de mieux définir les effets réels des corticostéroïdes sont nécessaires. Le but de ce mémoire a été d'étudier *in vivo* les effets de l'injection intra-articulaire de Triamcinolone hexacetonide sur les ménisques et le liquide synovial. Une étude préliminaire a été réalisée dans le but de connaître l'effet de l'âge sur ces deux éléments, afin d'assurer que les effets observés étaient bien dus aux corticostéroïdes et non pas à la différence d'âge entre les animaux. L'étude préliminaire n'a montré aucune corrélation significative entre l'âge des animaux et les différents paramètres étudiés (structure du ménisque, contenu en acide hyaluronique dans la synovie et activité des métallo-protéases matricielles MMP-2 et MMP-9 dans la synovie). L'injection de Triamcinolone hexacetonide n'a montré aucun effet significatif sur la composition biochimique des ménisques (contenus en eau, protéoglycanes et collagène) ou sur les caractéristiques du liquide synovial (concentration en acide hyaluronique dans la synovie et activité des métallo-protéases matricielles dans la synovie).

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Promoteur: J.-M. Vandeweerd

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Summary

Knee osteoarthritis is a disease characterized by a degradation of articular cartilage and synovial membrane inflammation that accelerates the degradation. This pathology has consequences on the whole joint, i.e. articular cartilage, menisci, synovial membrane, synovial fluid, ligaments and bones. One of the most common used treatment is the intra-articular injection of corticosteroids. Despite their massive use, the real effects of these drugs on the whole joint are not very well known. There is a need to perform studies allowing a better definition of corticosteroids effects. The goal of this master thesis was to study *in vivo* the effects of intra-articular injections of Triamcinolone hexacetonide on menisci and synovial fluid. A preliminary study was conducted to complement knowledge about the effects of ageing on menisci and synovial fluid to ensure that the corticosteroids effects observed thereafter were well due to the drugs and not to the age of the animals. The preliminary study did not show any significant correlation between age and the outcome measures (structure of menisci, hyaluronic acid content in the synovial fluid and matrix metalloproteinases activity in the synovial fluid). Intra-articular injections of Triamcinolone hexacetonide did not show any significant effects neither on the biochemical composition of menisci (water, proteoglycan and collagen contents) nor on the synovial fluid (hyaluronic acid content in the synovial fluid and matrix metalloproteinases activity in the synovial fluid).

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Abbreviations

Acan: aggrecan
ARRIVE: animal research: reporting of in vivo experiments
C4S: chondroitin 4-sulphate
C6S: chondroitin 6-sulphate
Col1A1: type-1 collagen
Col2A1: type-2 collagen
CS: corticosteroids
CTA: computed tomography arthrography
DC3: rapid decalcifier
DMBA: dimethylaminobenzaldehyde
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
GAG: glycosaminoglycan
HA: hyaluronic acid
IL-1: interleukin-1
MMPs: matrix metalloproteinases
MMP-2: matrix metalloproteinase-2
MMP-9: matrix metalloproteinase-9
MMP-13: matrix metalloproteinase-13
NF- κ B: nuclear factor-kappa B (transcription factor)
OA: osteoarthritis
OARSI: osteoarthritis research society international
OH-Pro: hydroxyproline
PGL4: proteoglycan 4
qRT-PCR: quantitative retro-transcription poly-chain reaction
SAPL: surface-active phospholipids
SDS: sodium dodecyl sulfate
TAE: tris-acetate-EDTA
TH: triamcinolone hexacetonide
TIMPS: tissue inhibitors of metalloproteinases

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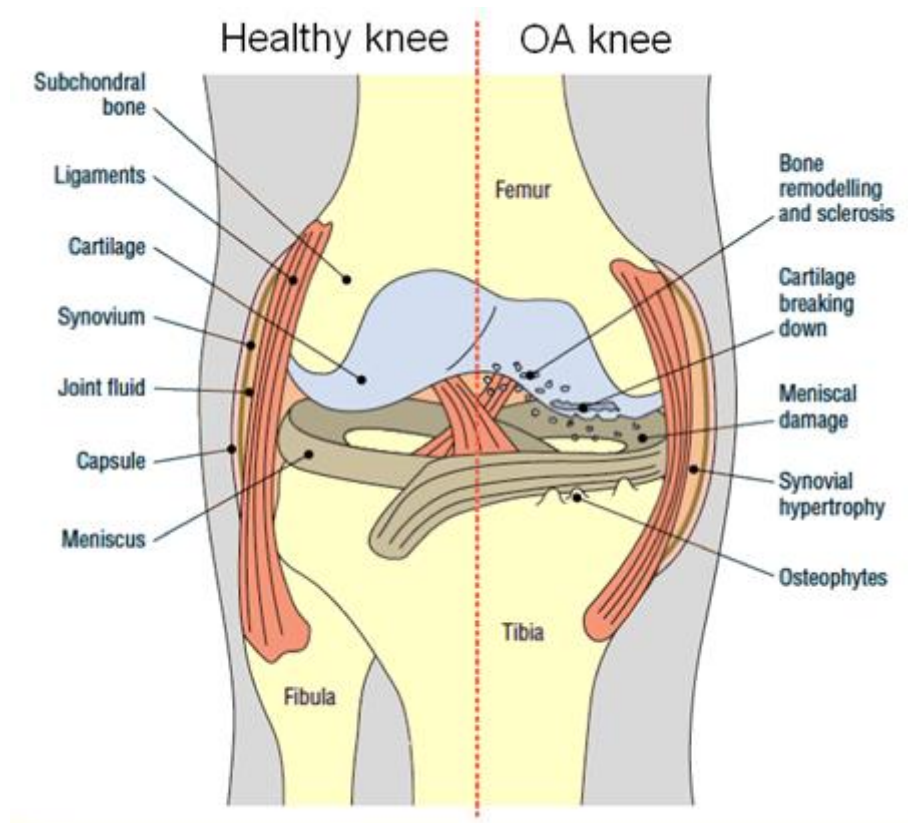


Figure 1: Comparison between a healthy and an osteoarthritic knee joint (*adapted from Poole, 2012*). The left side of the picture represents a healthy knee. Subchondral bone, ligaments, articular cartilage, synovium, synovial fluid and menisci constitute the joint. The right side represents an osteoarthritic (OA) knee. OA causes several damages including bone remodeling, cartilage and meniscal damages, synovium inflammation and osteophytes formation.

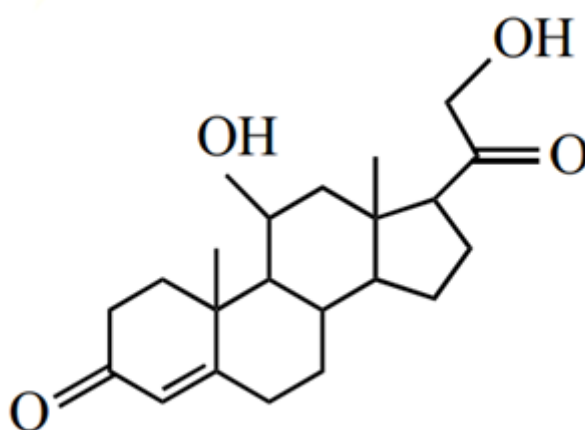


Figure 2: Biochemical structure of corticosteroid (Vinson, 2011). The picture represents cortisone, an endogenous glucocorticoid. The “pregnane” core is made of four fused rings: one with five carbon atoms and three with six carbon atoms.

I. INTRODUCTION

1. Osteoarthritis

1.1. Pathology: prevalence and mechanisms

It is estimated that more or less 10% of the world's population older than 60 years old suffer from osteoarthritis (OA) or from clinical problems associated with this pathology (Cooper et al, 2013). This degenerative and inflammatory disease of the synovial (diarthrodial) joints occurs when joint tissues respond inadequately to increased biomechanical constraints (Englund, 2010). It can affect hip and hand, and it is the most common pathology of the adult knee in man (Katsuragawa et al, 2010; Fibel et al, 2015). OA is also a cause of lameness in several animal species including horses (McIlwraith, 2010) and dogs (Sanderson et al, 2009).

OA is a disease of the articular cartilage though it is now well known that it can involve all other components of the joint i.e. menisci, bones, articular capsule, ligaments and synovium (Scanzello et Goldring, 2012) (**Figure 1**).

In an osteoarthritic knee, microscopic and macroscopic changes appear in the articular cartilage, in association with an inflammatory process. There is an imbalance between synthesis and degradation of the cartilaginous extracellular matrix and this leads to the release of cartilage fragments in the synovial cavity. Debris stimulate the release of pro-inflammatory mediators leading to synovial inflammation and sometimes synovial effusion (Bijlsma et al, 2011). The inflammatory state stimulates proteolytic enzymes that deteriorate the extracellular matrix and a vicious cycle is created (Lohmander et al, 2003). The modified extracellular matrix becomes more fragile and this causes structural changes and cartilage defects visible microscopically and macroscopically (**Figure 1**). Besides, it is reported that approximately 80% of osteoarthritic knees also have meniscal damages (Englund et al, 2009).

1.2. Treatment: zoom on corticosteroids injections

In addition to knee arthroplasty, non-pharmacological and pharmacological non-surgical strategies exist to treat OA (Ayhan et al, 2014). Examples of non-pharmacological therapies are self-management, exercises, weight reduction, and walking supports while examples of pharmacological strategies are non-steroidal anti-inflammatory drugs and corticosteroids (CS) (Maricar et al, 2013).

CS are a class of steroid hormones. They are organic compounds characterized by a core of four fused rings made of 17 carbon atoms. Three rings are made of six carbons and one is made of five carbons. Each CS owns a chemical core with supplementary specific functions (**Figure 2**). Currently, there are five injectable CS registered by Food and Drug Administration: methylprednisolone acetone, triamcinolone acetone, betamethasone acetone or sodium phosphate, dexamethasone and triamcinolone

hexacetonide (TH) (Ayhan et al, 2014).

These drugs are injected most of the time intra-articularly as prodrugs that need to be hydrolyzed to be active. This kind of injection delivers the drug locally and causes less systemic effects (Habib, 2009). CS act on nuclear steroid receptors to stop the inflammatory and immune cascade implicated in the cartilage degeneration found in OA (McIlwraith, 2010).

CS have anti-inflammatory and immunosuppressive effects (Ayhan et al, 2014). On the one hand, they prevent the activation, migration and accumulation of inflammatory cells. On the other hand, they inhibit phagocytosis and reduce capillary dilatation. They diminish the production of proteolytic enzymes and they inhibit the synthesis of inflammatory mediators, such as prostaglandins. They also reduce vascular permeability.

1.3. CS injections: lack of evidence

Since their first description in 1951, CS have been widely used (Ishikawa, 1981). In recent years, several reviews of the scientific literature about CS have been published (Bellamy et al, 2006; Bannuru et al, 2009; Hepper et al, 2009; Douglas, 2012).

Authors concluded that CS have only a short term effect on pain (4 to 6 weeks). Their dose regimen and the treatment duration are mostly determined according to clinician preferences. In addition, there is a debate on whether CS are beneficial or deleterious for the articular cartilage. Animal studies were generally mentioned to try to answer that question.

However, another recent systematic review of the literature showed that only 35 relevant controlled studies in animals have been performed on the CS effects between 1965 and 2014 (Vandeweerdt et al, 2015).

First animal studies were conducted in rabbits. Conclusions were that injections of hydrocortisone acetate disrupted the cartilaginous extracellular matrix in a dose dependent manner and that triamcinolone injections could cause histological changes of cartilage. High doses of CS were used in those studies. Moreover, the methodology was also particularly poor (Vandeweerdt et al, 2015).

In horses, studies about methylprednisolone acetonide showed it had deleterious effects. About triamcinolone acetonide, one study showed positive effects on the cartilage but did not take into account age and individual variations (Vandeweerdt et al, 2015).

In dogs, TH and methylprednisolone acetonide injections were reported to have positive effects on the reduction of osteophytes formation. However, a weakness of these studies was that the assessment of osteophytes sizes was not blinded (Vandeweerdt et al, 2015).

The systematic review concluded that those animal studies did not help to draw conclusions about the consequences of CS injections on articular cartilage. Studies were weakened by a lack of standardization (in term of number of animals, age and exercises performed), and by inappropriate study designs and methodologies.

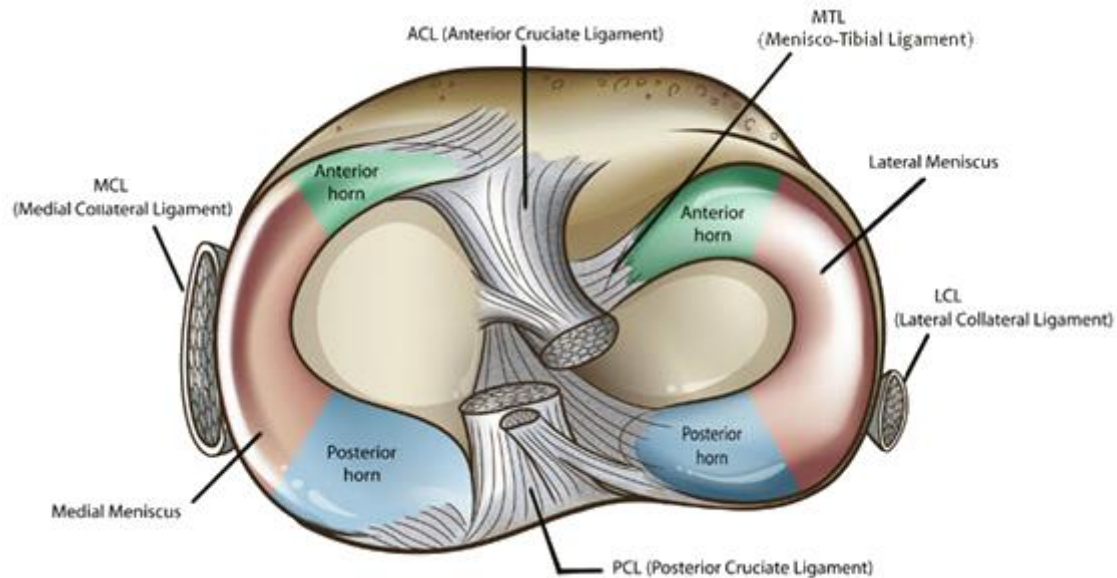


Figure 3: Dorsal view of the menisci (www.boneandspine.com). Both menisci are composed of a body (in red) and two extremities called horns (posterior horns are in blue and anterior horns are in green). Four menisco-tibial ligaments (MTL) link each horn to the tibial plateau. Cruciate ligaments (PCL and ACL) and collateral ligaments (MCL and LCL) are visible on the picture. They stabilize the joint though they allow a significant range of movement (Fox et al, 2012; Howell et al, 2014).

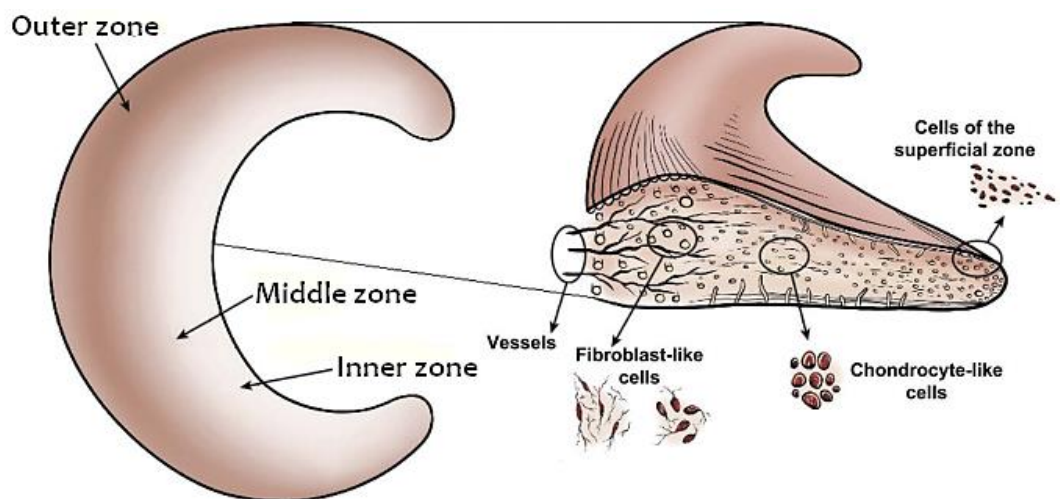


Figure 4: Regional variations in meniscal cell types (*adapted from* Makris et al, 2011). Cells in the inner and middle meniscal zones are chondrocyte-like cells. Their main function is to synthesize type-I collagen. Cells in the outer meniscus are fibroblast-like cells. They have cytoplasmic projections coming from the main body that facilitate communication with neighbor cells. Their main functions are to maintain the homeostasis establishing contacts with other cells and to respond to circumferential and compressive loadings. Just below the meniscal surface, there is also a minority of superficial cells.

The impact of CS on menisci is not better known despite the importance of these structures in the joint. Only two studies conducted in rabbits and dogs assessed potential meniscal changes caused by prednisolone acetate and halopredone acetate respectively (Ishikawa, 1981; Yamashita et al, 1986). CS effects on the synovial fluid are not vastly covered neither. Few previous studies made in man and horses have assessed the impact of CS on synovial fluid characteristics such as the hyaluronic acid (HA) content (Tulamo et al, 1991; Ronéus et al, 1993; Pitsillides et al, 1994) and the activity of several matrix metalloproteinases (MMPs) (Clegg et al, 1998; Shimizu et al, 2010; Knych et al, 2015).

As this master thesis focused on menisci and synovial fluid, the following sections provide relevant backgrounds about those components of the knee joint.

2. Menisci

Menisci are semicircular fibro-cartilaginous elements found in the knee. There are two menisci: one medial and one lateral. Both are located between the distal femur and the proximal tibia (**Figure 1**).

Menisci improve the congruency between the two femoral and tibial articular surfaces. They are submitted to axial, radial, and circumferential forces and act mainly by redistributing these contact forces across the tibio-femoral joint and by transmitting them over a larger surface area (Lee et Fu, 2000). Menisci have also roles in joint lubrication, shock absorption and proprioception (Fox et al, 2012).

2.1. Anatomy

The proximal meniscal surfaces are concave, allowing for articulating with the convex femoral condyles, while the distal surfaces are flat to match with the tibial plateau (Brindle et al, 2001; Fox et al, 2012). Both menisci are composed of a body and two extremities called “horns”. There are a series of ligaments linking the menisci to the bones (Fox et al, 2012) (**Figure 3**). In man, the medial meniscus is 3cm wide, 5cm long and covers between 51 and 74% of the tibial plateau while the lateral meniscus is 3cm wide, 4cm long and covers between 75 and 93% of the surface of the tibial plateau (Howell et al, 2014).

2.2. Composition

Menisci are made of fibrocartilage, i.e. a mixture of fibrous and cartilaginous tissues (Howell et al, 2014). The name “fibrocartilage” was given because of the partly fibrous appearance of the extracellular matrix observed with an optical microscope (Ghadially et al, 1983). Menisci are composed of different elements: cells and an extracellular matrix made in majority of collagen fibers and proteoglycans, minerals and water. These elements deserve description since they can be affected by CS injection.

2.2.1. Cells

The most abundant cell type in the inner and middle meniscus is fibro-chondrocytes (Howell et al, 2014) (**Figure 4**). They are not called chondrocytes because, contrary to articular chondrocytes that produce mostly type-II collagen, meniscal fibro-chondrocytes synthesize type-I collagen (Verdonk, 2010).

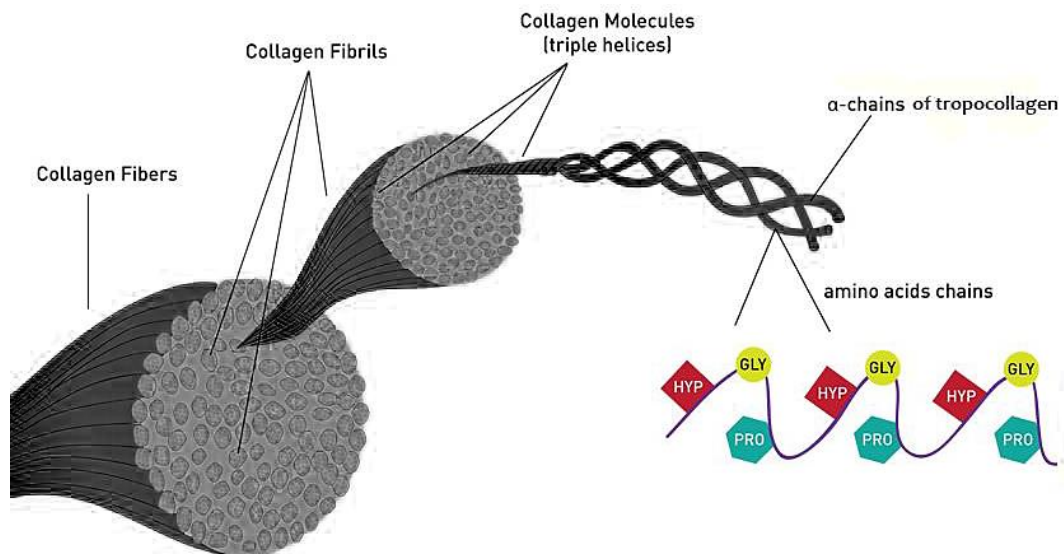


Figure 5: Schematic view of a collagen fiber (*adapted from <http://world.gold-collagen.com>*). The fiber is made of collagen fibrils. These are made of triple alpha right-handed super helices of tropocollagen. These helices are formed by three left-handed helices made of repeated sequences of three amino acids (Glycine-Proline-X or Glycine-X-Hydroxyproline).

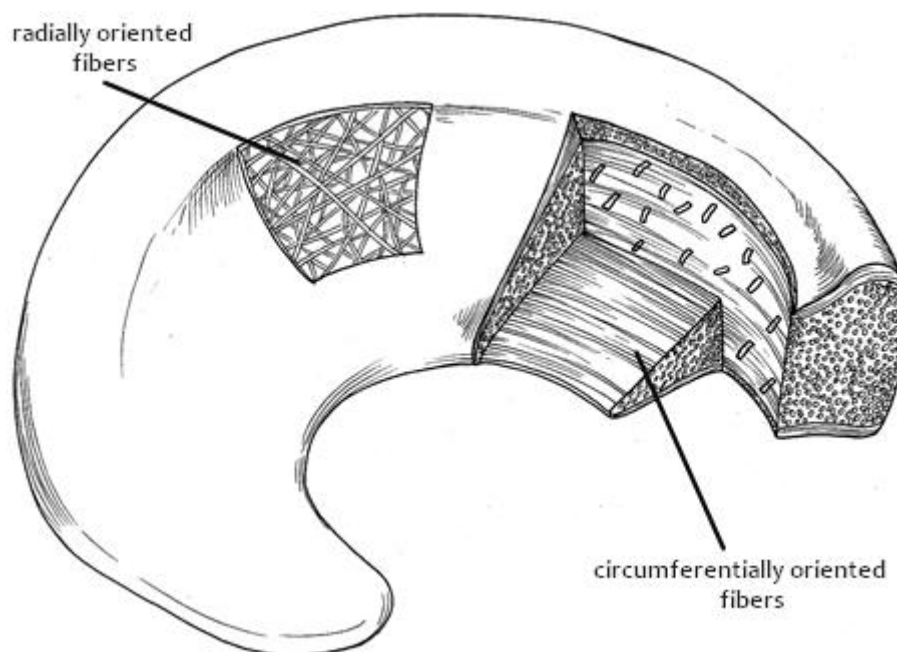


Figure 6: Schematic view of collagen fibers organization in the meniscus (*adapted from Greis et al, 2002*). Collagen bundles are radially oriented in the superficial layers while they are circumferentially oriented in the deep zones.

Fibro-chondrocytes are round and surrounded by an abundant pericellular matrix (Ghadially et al, 1983; Howell et al, 2014).

In the outer meniscus, cells look like articular fibroblasts and they are called fibroblast-like cells (Howell et al, 2014) (**Figure 4**). They are oval, fusiform and surrounded by a dense connective tissue but not by a pericellular matrix (Ghadially et al, 1983; Verdonk, 2010).

2.2.2. Extracellular matrix

Meniscal cells produce components of the extracellular matrix and ensure their turnover and their integrity (Fox et al, 2012; Howell et al, 2014). The extracellular matrix is mostly composed of collagen, proteoglycans and water.

Collagen fibers are responsible for the tensile strength of the menisci (Fox et al, 2012). Meniscal cells produce a precursor of collagen, procollagen, in their endoplasmic reticulum (**Figure 5**). After post-translational modifications, procollagen molecules are transported to the Golgi apparatus. They are excreted from the cells and their extremities are sectioned thanks to proteinases. The result is called tropocollagen. Tropocollagen molecules form fibrils stabilized by interfibrillar crosslinks thanks to the action of a specific enzyme, the lysyl oxidase. Finally, fibrils are assembled together into fibers of collagen (Viguet-Carrin et al, 2006) that have a diameter of 10-20 nanometers (Todhunter et Johnston, 2003).

There are numerous collagen types that can be differentiated according to their molecular composition and the kind of fiber they form (Bornstein et Sage, 1980; Burgeson et Nimni, 1992). Several types of collagen exist in varying quantities in each region of the meniscus. The majority of collagen is type-I in the outer meniscal zone (80%) but other types (-II, -III, -IV, -VI, and -XVIII) are present in smaller amounts. In the inner zone, the most dominant type is type-II (60%), followed by type-I (Makris et al, 2011). Consequently, type-I collagen is the predominant form in the meniscal fibrocartilage.

Collagen fibers are oriented circumferentially in the deeper layers of the meniscus while radially oriented fibers are in the superficial layers (Fox et al, 2012) (**Figure 6**). Biomechanical properties vary with the orientation of collagen fibers. The response of meniscal tissues is different in traction and compression depending on the area, i.e. superficial or deeper zones (Mcdermott et al, 2010).

Besides collagen fibers, there is also a combination of mature and immature elastin fibers in low concentration (<1%) (Makris et al, 2011). Elastin aids in the recovery of shape after a load deformation (Brindle et al, 2001).

Proteoglycans is the second-largest group of macromolecules present in the meniscal extracellular matrix (Fox et al, 2012). They consist of a core protein with covalently attached linear glycosaminoglycan chains. These chains are long unbranched polysaccharides made of repeating disaccharide units: the first is an uronic acid or a galactose, and the second is an amino sugar.

The glycosaminoglycans found in meniscus are chondroitin-6-sulfate (40%), dermatan-sulfate (20 to 30%), chondroitin-4-sulfate (10 to 20%) and keratin-sulfate (15%) (Fox et al, 2012).

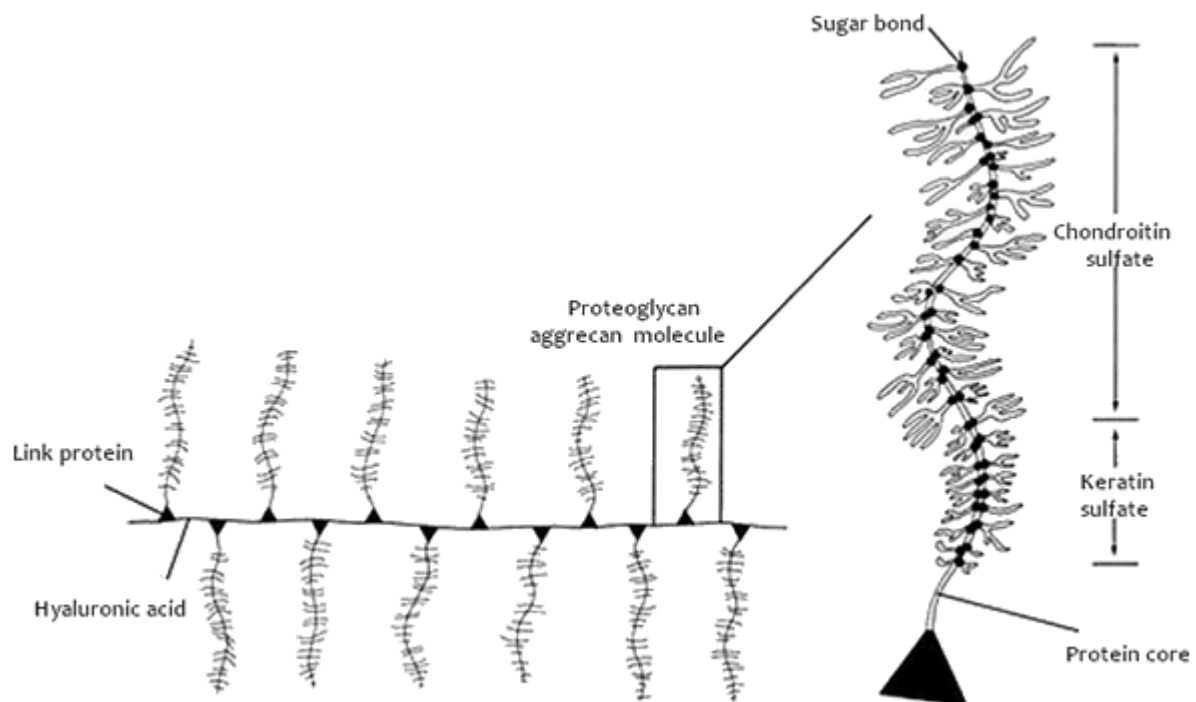


Figure 7: Schematic view of a major proteoglycan of the meniscus, aggrecan (adapted from <http://www.mobileappscentre.co.uk/healthapp/category/basic-sciences/cartilage>). Aggrecan is made of proteoglycan molecules composed of chondroitin-sulfate and keratan-sulfate glycosaminoglycans. Proteoglycan molecules are linked to “core” proteins attached to a hyaluronan molecule thanks to link proteins (Fox et al, 2012).

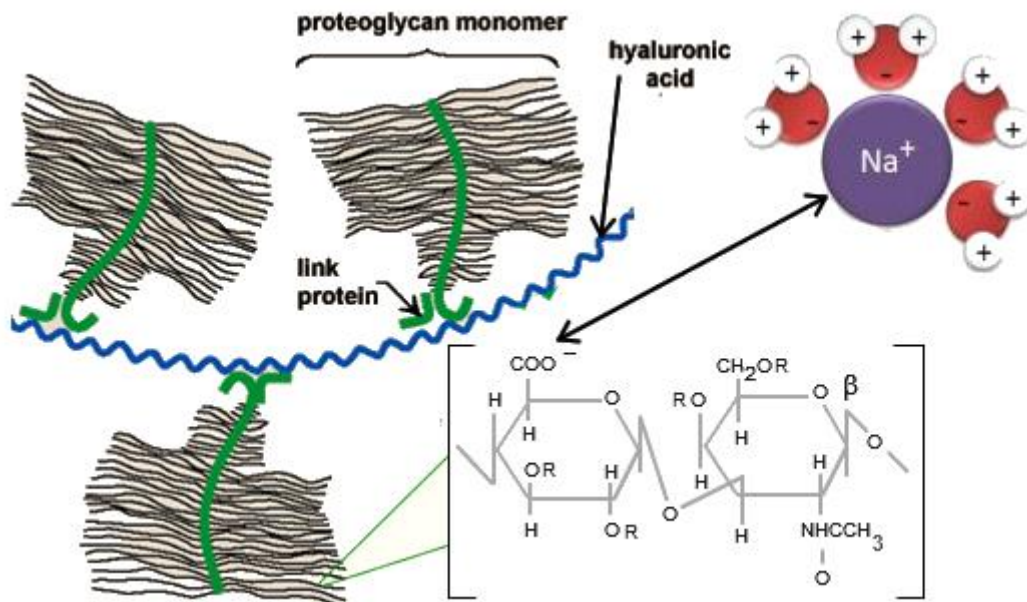


Figure 8: Interactions between proteoglycans and water (adapted from chemistry.tutorvista.com and imueos.blogspot.be). Proteoglycans are negatively charged because of the presence of glycosaminoglycan carboxyl groups (COO^-) and this property enables to interact with sodium. Then, electrostatic interactions between sodium ions and water molecules can occur. Sodium ion is in purple and water molecules are made of oxygen (in red) and hydrogen (in white) atoms.

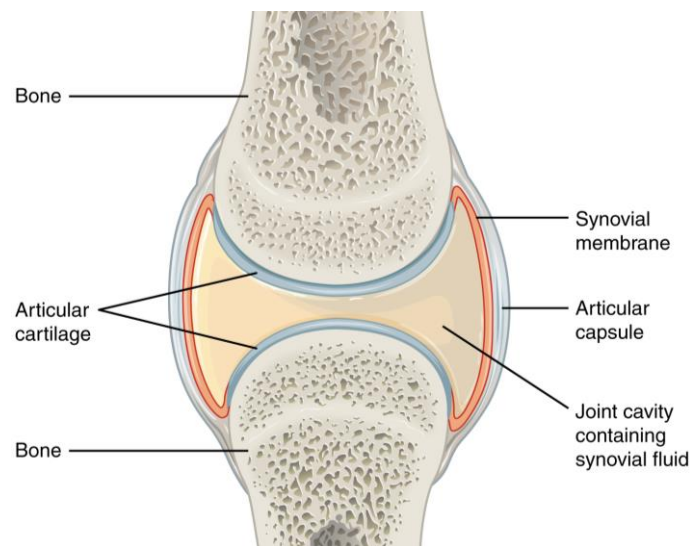


Figure 9: Schematic view of a synovial joint (<http://philschatz.com/>). The articular capsule covers the joint cavity containing synovial membrane and synovial fluid.

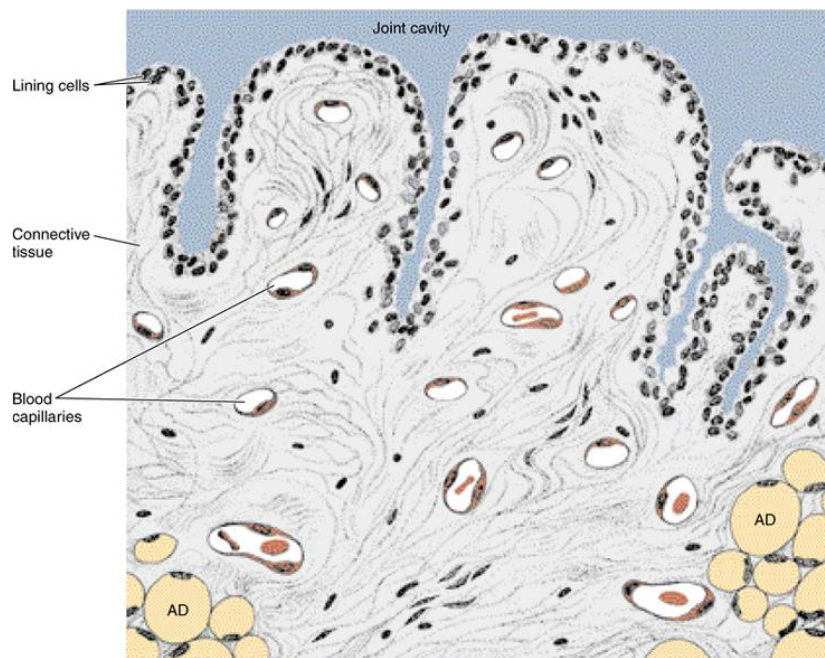


Figure 10: Schematic view of the synovium (Nadine Antoine). The joint cavity containing the synovial fluid is in blue. Two layers of lining cells are visible. Deep to these layers, a loose connective tissue contains blood capillaries and adipocytes (AD). The synovium is arranged in villi but it could also be flat.

Aggrecan, the most abundant proteoglycan, is made of keratin-sulfate and chondroitin-sulfate chains. It can interact with another important component of the extracellular matrix, HA, to form macromolecular aggregates enabling the resilience of the menisci (Pearle et al, 2005; Fox et al, 2012) (**Figure 7**). Other proteoglycans are present: biglycan and decorin, which are chondroitin-sulfate and dermatan-sulfate proteoglycans (Scott et al, 1997).

Proteoglycans are negatively charged because of the presence of glycosaminoglycan carboxyl groups and this property enables the interaction with cations, such as sodium. In turn, sodium can attract water (Lu et Mow, 2008) (**Figure 8**).

Matrix glycoproteins are also present in the meniscus. They are mainly located around the collagen bundles in the inter-territorial matrix. These matrix molecules include link proteins, which stabilize proteoglycan-hyaluronan aggregates, and adhesion glycoproteins, which binds other extracellular matrix components or cells (Makris et al, 2011; Fox et al, 2012; Howell et al, 2014).

Finally, water is an essential component of the meniscus. Three quarter of the meniscus is water (Makris et al, 2011) which is free or retained by interactions involving minerals and proteoglycans.

3. Synovial fluid

The knee joint is part of a category called “synovial joints”. This is the most mobile type of joints in mammals. This involves lubrication made by a fluid filling the articular cavity, called synovial fluid (**Figure 9**). In healthy knees, synovial fluid is pale yellow and viscid (Lipowitz et al, 1985). It is produced by the synovium, a thin membrane made of synovial cells, called synoviocytes (Tamer, 2013).

3.1. Production of synovial fluid by the synovial membrane

The normal synovium is made of 1 to 4 layers of cells which are mixed in deep zones with fibrocollagenous tissue containing several cellular types such as fibroblasts, macrophages and adipocytes. The synovial membrane has important blood supply through this loose fibrocollagenous tissue (Wenham et Conaghan, 2010). The surface of the synovium can be flat or arranged in villi (**Figure 10**).

Synovial cells are non-vascular and non-epithelial cells (Schneider, 2007). Two different subtypes (A and B) of synoviocytes are found. Subtype A cells look like macrophages and their main role is to phagocyte undesirable substances from the synovial fluid. Subtype B cells look like fibroblasts and their role is to secrete substances forming the synovial fluid, such as HA and lubricin (Shikichi et al, 1999). Synovial fluid is a plasma dialysate modified by the substances produced mainly by type B synoviocytes (Lipowitz, 1985).

The synovial membrane acts as a semipermeable membrane which controls the molecular traffic in and out of the joint: high molecular weight lubricating molecules such as HA have to stay inside the joint cavity (Scanzello et goldring, 2012).

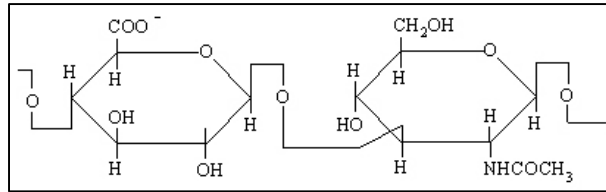


Figure 11: Structure of hyaluronan (Nekas et al, 2008). It is made of repeating disaccharide units formed by N-acetyl-d-glucosamine and d-glucuronic acid. HA structure is very stable and its size can reach 10^7 Daltons.

3.2. Composition

The difference between this fluid and other fluids derived from plasma is the high content in HA, which, in combination with the glycoprotein lubricin, reduce frictions in the joint and maintain the integrity of the articular surfaces (Lipowitz, 1985).

HA, the main component of the synovial fluid lubrication system, was first isolated from the vitreous body of cows' eyes in 1934 (Nekas et al, 2008; Inoue et al, 2011). It is a linear non-sulfated glycosaminoglycan secreted in the salt form (hyaluronate) into the joint. It is synthesized by hyaluronan synthases in the cell plasma membrane rather than in the Golgi and it is made of repeating disaccharide units formed by N-acetyl-d-glucosamine and d-glucuronic acid joined thanks to alternating β -1,4 and β -1,3 glycosidic bonds (**Figure 11**, Nekas et al, 2008).

In the olden days, HA was only considered as an inert space filling substance able to surround itself with water to maintain joint structure and to prevent dehydration (Petrey et De la motte, 2014). Thanks to hydrogen bonding between the hydroxyl groups along the HA chain, the structure, which is in the form of a random coil in solution, can trap about a thousand times its weight in water (Nekas et al, 2008). But joint lubrication is not the only role of this glycosaminoglycan. In the synovial fluid, HA network acts as a barrier retarding the diffusion of other molecules and regulating their transport through the intercellular spaces (Tamer, 2013). It also has a role in inflammation because it inhibits the migration of inflammatory cells, the production of MMPs and pro-inflammatory mediators (Berbis, 2010; Plickert et al, 2013).

Minor components of the synovial fluid are proteoglycan 4 (PGL4), lubricin and surface-active phospholipids (SAPL) (Blewis et al, 2007; Tamer, 2013). The fluid also contains glucose, proteins, and traces of proteolytic enzymes (Volk et al, 2003). The main cell type found in the synovial fluid is lymphocytes. Monocytes, macrophages and neutrophils are rarely seen but their number increase in case of disease (Perman, 1980).

3.3. Functions

Synovial fluid has two main roles. It brings nutrients to the articular cartilage chondrocytes because cartilage is not vascularized and it does not have any blood supplies. Glucose, which gives energy to the chondrocytes, is transported from the periarticular vasculature to the cartilage thanks to the synovial fluid (Faires and Mccarty, 1962).

The second role is to lubricate the knee joint. The fluid helps to reduce frictional resistance between the two articular surfaces. The lubrication property is directly related to the concentration and molecular weight of HA (Lipowitz, 1985).

4. OA changes in menisci and synovial fluid

Though it was reported that OA joints could lead to changes in menisci, these changes are variable and still poorly understood. OA could disrupt the meniscal extracellular matrix decreasing the cellularity, modifying collagen and GAG contents and impairing meniscal

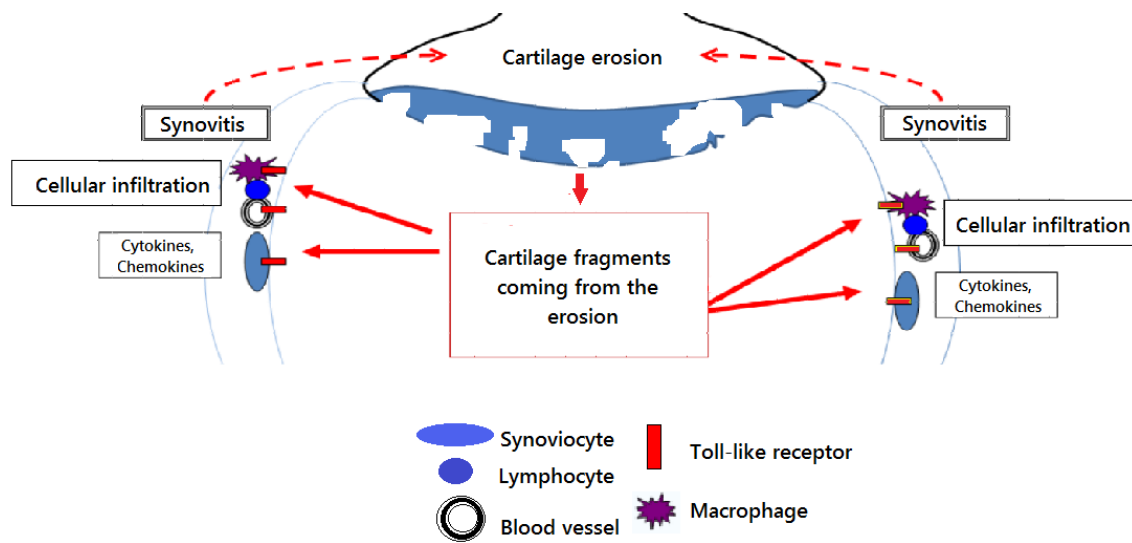


Figure 12: Mechanisms of synovitis (modified from Scanzello et Goldring, 2012). Cartilage fragments due to erosion cause the activation of cytokines and chemokines in the synovial membrane. They also cause infiltration of inflammatory cells. Synovitis can cause cartilage degradation. A vicious circle is created and maintains the inflammation state.

Subgroup	MMP	Name	Substrate
1. Collagenases	MMP-1	Collagenase-1	Col I, II, III, VII, VIII, X, gelatin
	MMP-8	Collagenase-2	Col I, II, III, VII, VIII, X, aggrecan, gelatin
	MMP-13	Collagenase-3	Col I, II, III, IV, IX, X, XIV, gelatin
2. Gelatinases	MMP-2	Gelatinase A	Gelatin, Col I, II, III, IV, VII, X
	MMP-9	Gelatinase B	Gelatin, Col IV, V
3. Stromelysins	MMP-3	Stromelysin-1	Col II, IV, IX, X, XI, gelatin
	MMP-10	Stromelysin-2	Col IV, laminin, fibronectin, elastin
	MMP-11	Stromelysin-3	Col IV, fibronectin, laminin, aggrecan
4. Matrilysins	MMP-7	Matrilysin-1	Fibronectin, laminin, Col IV, gelatin
	MMP-26	Matrilysin-2	Fibrinogen, fibronectin, gelatin
5. MT-MMP	MMP-14	MT1-MMP	Gelatin, fibronectin, laminin
	MMP-15	MT2-MMP	Gelatin, fibronectin, laminin
	MMP-16	MT3-MMP	Gelatin, fibronectin, laminin
	MMP-17	MT4-MMP	Fibrinogen, fibrin
	MMP-24	MT5-MMP	Gelatin, fibronectin, laminin
	MMP-25	MT6-MMP	Gelatin
6. Others	MMP-12	Macrophage metalloelastase	Elastin, fibronectin, Col IV
	MMP-19		Aggrecan, elastin, fibrillin, Col IV, gelatin
	MMP-20	Enamelysin	Aggrecan
	MMP-21	XMMP	Aggrecan
	MMP-23		Gelatin, casein, fibronectin
	MMP-27	CMMP	Unknown
	MMP-28	Epilysin	Unknown

Figure 13: MMPs classification according to their structure and their substrate preference (Snoek-van Beurden et Von den Hoff, 2005). The first column lists the six existing subgroups. The two following columns represent the metalloproteinase names. The last column contains the substrates that are digested by the enzymes.

tissues (Pauli et al, 2011). Collagen synthesis could also be modified (Katsuragawa et al, 2010).

In OA, synovium can be affected by cartilage fragments which enter in contact with the membrane, due to the matrix degradation. These matrix fragments can activate the innate immune response via toll-like receptors. Then, transcription factors are activated, with NF- κ B playing a prominent role. An inflammatory state is triggered and the synovium becomes a major source of pro-inflammatory soluble potent mediators (cytokines and chemokines) and catabolic enzymes able to degrade the extracellular matrix and to cause tissue damages. Synovial macrophages and white blood cells are activated and they promote the production of inflammatory molecules by chondrocytes, synoviocytes and macrophages themselves. This pathology is called synovitis (**Figure 12**) and its severity increases with the advancing stage of OA (Scanzello et Goldring, 2012).

Microscopically, several synovial abnormalities can be seen. These abnormal signs include infiltration of immune cells, fibrosis, villi hyperplasia/thickening of the synovial membrane and increased vascularity. In more severe cases of synovitis, cell infiltration can lead to the apparition of lymphoid aggregates (Wenham et Conaghan, 2010). The permeability of the membrane becomes altered and it disrupts the concentration of HA and lubricin in the joint. Therefore, the viscosity changes (Scanzello et Goldring, 2012).

Changes also occur in the composition of the synovial fluid. Soluble pro-inflammatory mediators and catabolic enzymes, such as aggrecanases and MMPs, are produced to degrade the cartilage extracellular matrix (Gepstein et al, 2002; Wenham et Conaghan, 2010). In case of synovitis, the expression and the activation of MMPs seem to increase (Scanzello et Goldring, 2012). It is presumed that CS injections could have an anti-inflammatory effect on these changes (Wenham et Conaghan, 2010).

In humans, more than 20 MMPs exist (Gepstein et al, 2002). They are classified in six groups, depending on their structure and substrate preference (**Figure 13**). Among these groups, the gelatinase group includes MMP-2 and MMP-9. These enzymes digest gelatin, the denatured form of collagen and their activity can be assessed by gelatin zymography. Studying these two MMPs could give insights about the inflammatory effect of CS on the knee joint (Snoek Van Beurden et Von den Hoff, 2005).

HA content in the synovial fluid is also modified in case of synovitis (Inoue et al, 2011). In healthy synovial fluid, the polysaccharide has a high concentration and a high molecular weight (Petrey et De la Motte, 2014). In case of inflammation, it becomes degraded by hyaluronidases (endoglycosidases) and the fragmentation leads to fragments of different sizes. Its concentration is also disrupted and has tendency to decrease (Liu et al, 2012). It is in association with a loss of the viscosity properties (Tamer, 2013).

It has been proved that HA has different effects on the inflammation in function of the polymer size (Petrey et de la Motte, 2014). However, these effects are not yet fully understood (Misra et al, 2015). Native large fragments seem to act as tissue integrity signals which suppress the inflammatory response. They also inhibit angiogenesis by preventing the formation of capillaries because they reduce the proliferation and migration of endothelial

cells (Tamer, 2013). They maintain water homeostasis and the matrix structure (Misra et al, 2015). On the contrary, low molecular weight fragments obtained in case of synovitis have pro-inflammatory effects. They activate macrophages and the expression of pro-inflammatory genes (Tamer, 2013; Petrey et De la motte, 2014; Misra et al, 2015).

II. OBJECTIVES

OA is the most common disease of the adult knee joint worldwide (Fibel et al, 2015). Intra-articular CS injections are frequently used to treat this pathology. However, only very few studies in humans and in animal models have assessed the effects of these drugs on the articular cartilage, menisci and synovial fluid. Whether CS injections are deleterious or beneficial for the knee joint is a question of debate. This master thesis attempted to fill in this lack of evidence by assessing the effect of CS on menisci and synovial fluid in an ovine model.

Some variables have to be taken into account in *in vivo* animal studies (Vandeweerd et al, 2015). Although gender, diet and breed can be standardized in research studies using large animals, it is not always possible to enroll animals of the same age. This is why the objective of the first part of the master thesis was to provide knowledge about the effect of age on the menisci and on the synovial fluid by performing a cross-sectional preliminary study within a population of research sheep.

The second and main objective of this master thesis was to assess, in the ovine model, the impact of a clinical dose (25 mg) of triamcinolone hexacetonide (TH) on the biochemical composition of menisci and on several synovial fluid parameters (HA content, HA fragments size and MMP-2 and -9 activities). TH has been chosen because it has been reported to have protective effects on articular cartilage (Vandeweerd et al, 2015). Moreover, it is the most insoluble CS and it has the longest duration of action (McIlwraith, 2010).

Criteria	Observations	Score
Comfort	Awake, interested in surroundings, chewing cud, eating	0
	Awake, not interested in surroundings, not chewing cud, reduced appetite	1
	Lethargic, depressed appearance, ears drooped, not chewing cud, not eating	2
	Head down, very lethargic, ears stay drooped when aroused, not chewing cud, grinding teeth	3
	Recumbent, no response when approached, fixed look or eyes half closed, grinding teeth	4
Movement	Normal ambulation, full weight-bearing, no lameness	0
	Slight lameness on the limb, toe-touching on all steps	1
	Lameness on the limb, toe-touching on some, but not all steps	2
	Lameness on the limb, not toe-touching on all steps when walking voluntarily, but will toe-touch when herded	3
	Lameness of the limb, not toe-touching in all steps when walking voluntarily and when herded	4
Flock behavior	Normal, moves with the flock	0
	Lethargic or lags behind the flock when flock is moved, but eventually joins them voluntarily	1
	Lags behind rest of the flock when flock is moved, but eventually joins them if encouraged to do so	2
	Always separated from the flock	3
Total score		0-11

Figure 14: Scale used to assess basic welfare parameters in sheep (Shafford et al, 2004). The well-being evaluation was performed by an experienced staff.

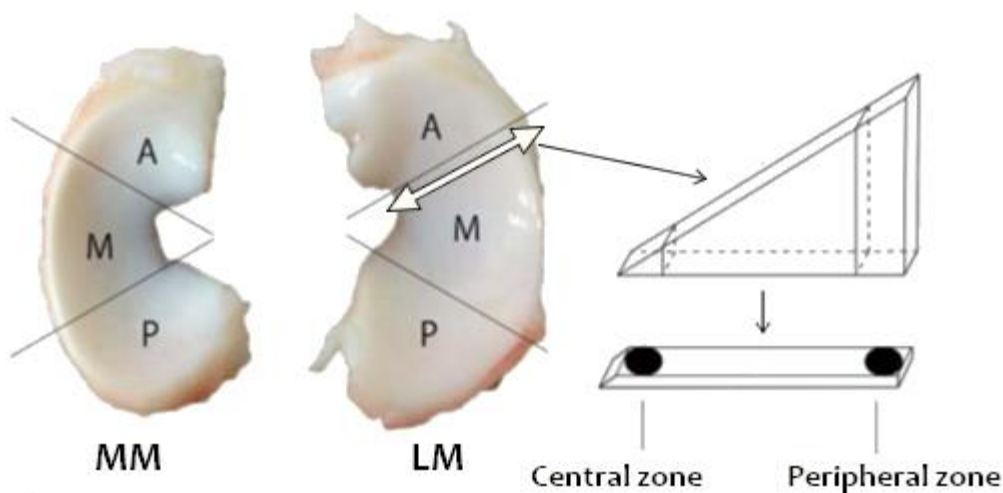


Figure 15: Schematic view of the division made into the menisci to assess meniscal histology and biochemistry (Zhang et al, 2014). MM: medial meniscus; LM: lateral meniscus; A: anterior part; M: middle part; P: posterior part. Cross-sectional samples harvested from the fibrocartilaginous tissues were cut about 0.5 cm thick. On the schema we can see central and peripheral zones of the cross-section.

III. MATERIAL AND METHODS

1. Foreword

1.1. Ethical statement

The experimental protocols were approved by the local ethical committee for animal welfare. All interventions were performed under general anesthesia. Animals did not feel pain, except during the introduction of an intravenous catheter for anesthesia. Sheep well-being was assessed thanks to a validated scale (Shafford et al, 2004) (**Figure 14**). The evaluation was performed by an experienced staff. This master thesis was in line with the “Animal Research: Reporting of In Vivo Experiments” (ARRIVE) guidelines for reporting animal research (Kilkenny et al, 2011).

1.2. Animals

The ovine model was used in this research project. Animal models are useful to study orthopedic diseases occurring in humans, and especially OA pathophysiology (Aigner et al, 2010). Human menisci look very similar to those in sheep (Saadinam et al, 2014). Sheep are large animals with orthopedic biomechanics comparable to that of humans (Burger et al, 2007), despite the fact they are quadrupeds.

In this study, sheep came from the Ovine Research Centre of the University of Namur. All were retired animals, due to mastitis or metritis but not because of musculoskeletal disease. During the study, sheep were kept in small groups in an outdoor paddocks and interior regulatory shelter (covered with straw) in the research center. The paddocks enabled a pleasant pasture and hay was permanently available.

1.3. Selection criteria

Sheep used in this research had no clinical signs of orthopedic disease at baseline. This was confirmed by clinical evaluation: they had no joint swelling or effusion, no pain or heat at palpation and no lameness when their locomotion was assessed.

2. First part: preliminary prevalence study

Age is an important variable in many diseases such as OA. In consequence, it is important to know whether that variable could influence the parameters measured in this master thesis in the research population that will be used.

2.1. Material

The study was conducted on samples (of meniscal tissues and synovial fluid) collected in 37 healthy crossed Texel ewes, euthanized between May 2013 and August 2013. They had been collected after euthanasia and stored at – 80° for analysis. For histological assessments, middle parts of menisci were used (parts “M” on **Figure 15**). Samples were transferred in a solution of 70% ethanol.

Criteria	Observations	Score
Tissue architecture, tissue loss	Normal	0
	Minimal disruption	1
	Moderate disruption with loss of tissue	2
	Complete loss of tissue architecture, >50% loss	3
Cell and matrix content and morphology	Normal	0
	Minimal alterations in cell and matrix content and morphology	1
	Moderate alterations in cell and matrix content and morphology	2
	Severe loss of cells, proteoglycans and collagen	3
Proliferative response	None	0
	Minimal proliferation of cells at synovial-meniscal junction	1
	Proliferation of cells at synovial junction and extending into tissue or along surfaces	2
	Marked proliferation of cells involving majority of remaining tissue	3
Total score		0-9

Figure 16: Table showing the microscopic grading system of meniscal histological alterations created by the OARSI (Cook et al, 2010). The table defines three criteria: tissue architecture and tissue loss, cell and matrix content and morphology, and proliferative response. Scores are from 0 to 3 for each criteria according to the severity of the alteration. The total score is between 0 and 9.

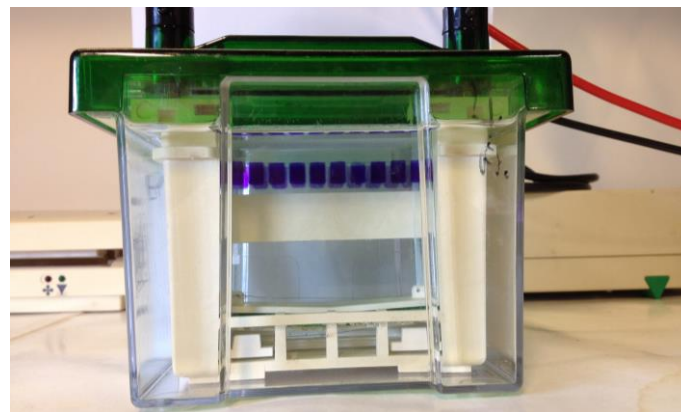


Figure 17: MInI-PROTEAN 3 system. Samples and standards are loaded in the wells made thanks to the comb. They appear in blue because dilutions were made thanks to the staining Laemmli. The picture was taken just before the electrophoresis migration of two hours.

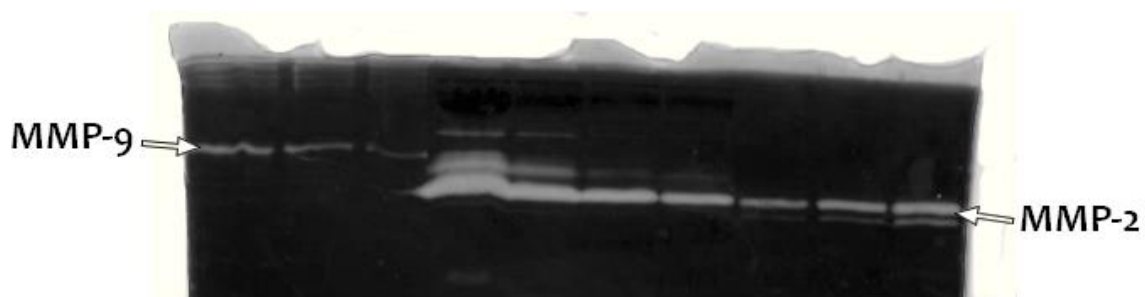


Figure 18: Zymography gel scanned and analyzed with the software ImageJ. Standards of MMP-9 (92 kDa) are in the three first wells. Then, the four following wells are filled in by four different dilutions of one sample. The last three wells contain standards of MMP-2 (62 kDa).

2.2. Methods

2.2.1. Histology of meniscus

Samples were decalcified in DC3 (solution made of nonionic surfactants, hydrochloric acid, EDTA) for 2 days and embedded in paraffin. Meniscal sections were performed with microtome and stained with Toluidine blue to reveal proteoglycan and glycosaminoglycan contents (Pauli et al, 2011; Sandmann et al, 2013).

Scores were given for the middle part of each meniscus. The examination was performed by two blinded investigators according to a scoring system published by Cook et al. (Cook et al, 2010; **Figure 16**). Histological slices were observed with an optical microscope at a magnification of 100 times.

2.2.2. HA dosage in the synovial fluid

HA was measured by an ELISA-like system. A miniplate was incubated with capture reagent overnight. Afterwards, the miniplate was washed and a block buffer was added to block the capture reagent for 1 hour. Samples were diluted and incubated in the reagent diluent for 2 hours, and then samples and standards were added into the plate.

After an incubation of 2 hours with a biotin associated detection reagent and another incubation of 20 minutes with streptavidin linked with a peroxidase, substrate solution was added and incubated for 20 minutes before adding the block solution. The plate was read immediately at 450 and 570 nanometers with a spectrophotometer to avoid disruptions due to the light (the substrate is chromogenic). For each measure, triplicates were done.

2.2.3. MMP-2 and MMP-9 activities assessments in the synovial fluid

Gelatin zymography was used to quantify the activities of the matrix metalloproteinases MMP-2 and MMP-9. This electrophoretic technique enables direct comparisons between potential activities of all samples (Volk et al, 2003). Samples and standards were added into a Mini-PROTEAN 3 system (**Figure 17**, Bio-Rad) made of two gels: one separation gel (containing gelatin) and one compression gel. Both gels contained 10% sodium dodecyl sulfate (SDS).

Gels were submitted to 2 hours of electrophoresis migration, during which the SDS denatured and made inactive the MMPs. Afterwards, gels were removed from the system and washed with Triton X100. Triton caused the partial enzymatic renaturation and activity recovery. Subsequently, gels were incubated in an activation buffer overnight at 37°C. During this incubation, renatured MMPs digested the gelatin substrate. After minimum 18 hours, gels were washed and colored. The staining was made of methanol, acetic acid and Coomassie blue. Then gels were discolored, scanned and analyzed with the software ImageJ (**Figure 18**). Curves of calibration were used to see the quality of the results. For each metalloproteinase, three standards were used to assess results accuracy.

2.3. Statistical analysis

Data were collected in Microsoft Excel and analyses were performed using R Studio. Kolmogorov-Smirnov tests were used to examine the normality of data. Due to the non-

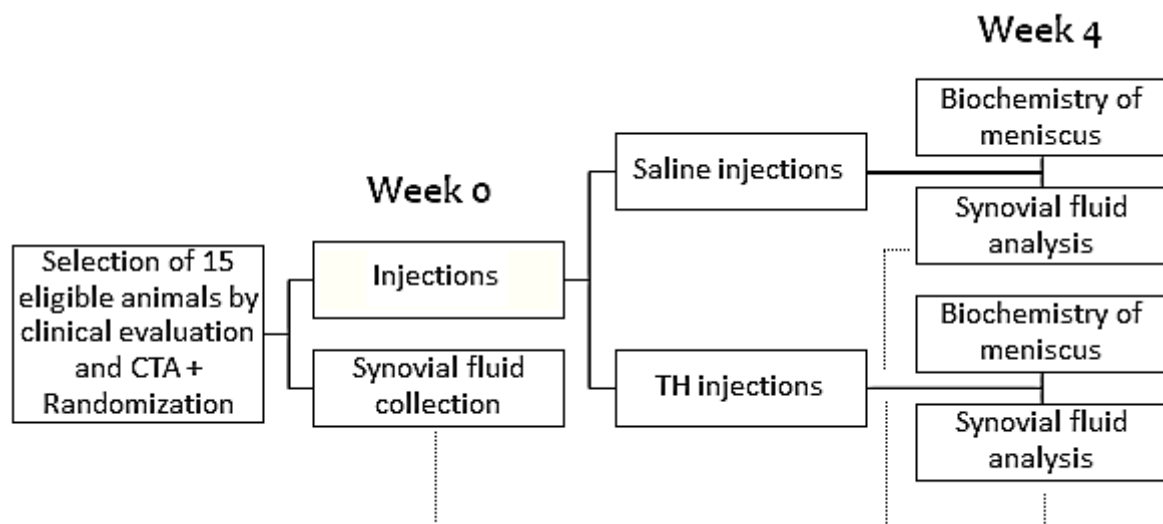


Figure 19: Study design. 15 animals were considered as eligible after clinical evaluation and computed tomography arthrography (CTA). Stifle joints were randomized and synovial fluid was collected at baseline. Then, injections were realized (either saline injections or TH injections). After 4 weeks, sampling was performed, allowing the assessment of the meniscal biochemistry. Synovial fluid was also collected after injections to perform analysis.

normality of data, the non-parametric Mann-Whitney-Wilcoxon test was performed to assess the independence of the results (lateral *versus* medial menisci and left *versus* right limbs). Regression linear models were performed to assess potential correlation between age and the different parameters of the study (histological scores, HA content and MMPs activities). A p-value less than 0.05 was considered as statistically significant.

3. Second part: study of the effects of triamcinolone hexacetonide

3.1. Study population and study design

15 healthy crossed Texel ewes were included in the study.

To study the effects of the CS on menisci and synovial fluid, a controlled trial was designed (**Figure 19**). Right or left stifle joints were randomly assigned into two groups. The first group was the control group (joints injected with saline: sodium chloride 0.9%) and the second group was the treatment group (joints injected with 25mg of TH: Hexatrione 2%).

The assignment of stifle joints into both groups was made using the software “Randomization”. Allocation to the groups and injections were performed by a clinician not involved in the experimental outcomes and all experimental procedures were conducted without knowing the repartition of the injections.

3.2. Animal selection

It is important to ensure that knees that will be compared are similar at baseline (absence of disease, similar stage of defects). Computed tomography arthrography (CTA) is accurate to assess cartilage structure (Hontoir et al, 2014). This technique uses contrast medium and computed tomography to detect cartilage/meniscal defects with a sufficient sensitivity and specificity.

After disinfection of the stifle, a solution made of 7ml of contrast medium (320mg/ml ioxalate meglumine and sodium ioxalate, Herbarix 320) and 13ml of saline was injected in the ovine femoro-patellar compartment using a paraligamentous technique. The assessments were made using an Emotion 6 CT (Siemens) and the acquisition protocol was: 130KV, 80mAs, pitch 0.4, collimation 0.63mm and rotation time of the tube 0.6s. Coronal, sagittal and transversal images were obtained and transferred on a medical software (PACS, TELEMIS). They were analyzed by an experienced radiologist. Only animals with healthy joints (without lameness, inflammation, major cartilage defects, osteophytes, subchondral bone changes, and meniscal abnormalities) were selected for the study.

3.3. Experimental procedures

3.3.1. Anesthesia

Animals were under general anesthesia for CTA and CS injections. To decrease the risk of errors (stomach liquid into the airways) and bloats (gas accumulation in stomach distended), sheep were put on a diet but water remained available.

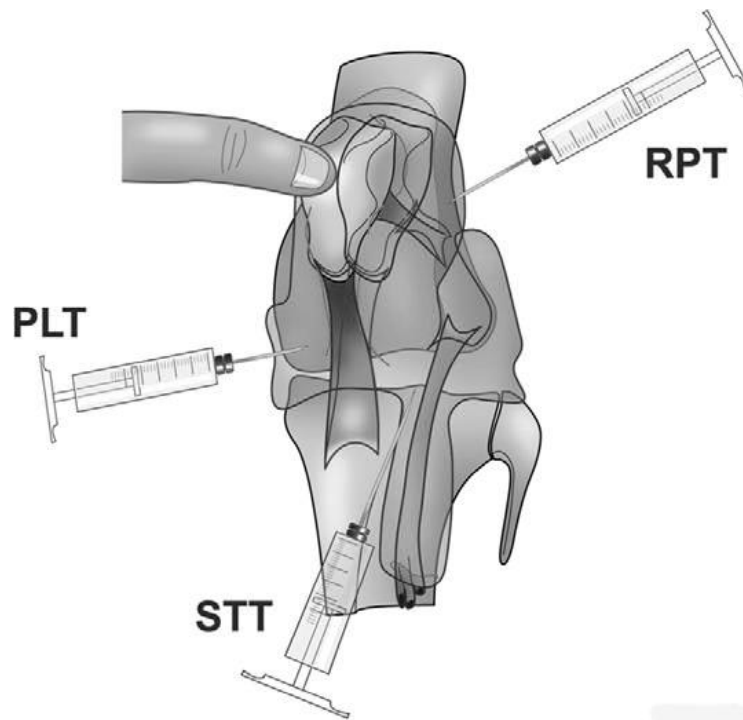


Figure 20: Schematic view of the needles' insertion into the knee joint during synovial fluid collection and injections (Vandeweerd et al, 2012). RPT: retropatellar technique; PLT: para-ligamentous technique; STT: subtendinous technique.

Before anesthesia, an intravenous catheter was introduced in the cephalic vein. The sedation was made thanks to 150µg/kg xylazine and 150µg/kg diazepam and the anesthesia was performed with IV pentobarbital at a dose of 3mg/kg for induction and 10mg/kg/h for maintenance (Goossens et al, 2013).

After induction of anesthesia, sheep were placed in sitting position with mouth open with a laryngoscope and an endotracheal tube was placed. They received oxygen at a volume of 6 liters per minute. Every 5 minutes, several parameters were recorded such as heart and respiratory rates, and the depth of anesthesia (by assessing corneal reflexes).

3.3.2. Injections

TH injections were performed one week after CTA scans. Anesthetized sheep were placed in dorsal recumbency. The stifle region was shaved and disinfected and the needle was inserted thanks to a para-ligamentous technique (**Figure 20**, Vandeweerd et al, 2012). Synovial fluid was collected if possible and then 1.25ml of solution (saline for the control group and 25mg of Hexatrione 2% for the treatment group) was injected in the joint. Sheep were monitored until recovery.

3.3.3. Euthanasia

As a previous study concerning the kinetic data of CS in human showed that the absorption of the drug was complete after a period of 2 to 3 weeks (Derendorf et al, 1986), sheep were euthanized 4 weeks after injections (because there was no need to keep them alive since the drug was potentially already absorbed). Euthanasia was made thanks to intravenous 150mg/kg administered pentobarbital. After death, hind limbs were disarticulated at the level of the coxo-femoral joint. Collection of samples took place within 2 hours of euthanasia.

3.3.4. Sampling

For all samplings (both in the preliminary study and in the major study), synovial fluid was collected if possible after sheep euthanasia using a retropatellar technique (**Figure 20**, Vandeweerd et al, 2012). The knee joints were disarticulated at the level of the coxo-femoral joint. Articular capsules were removed from the limbs and opened. Meniscal peripheral extremities were detached from the capsules and menisco-tibial ligaments were transected. Horn bony attachments were removed. Menisci were collected and meniscal samples were obtained in the middle part of the menisci (part “M” on **Figure 15**). All samples were stored at -80° for analysis.

3.4. Experimental outcomes

3.4.1. Biochemistry of meniscus

Freeze drying and papain digestion

Meniscal samples were freeze dried for 48h (AMSCO/Finn-Aqua Lyovac GT 2, pressure = 6.5×10^{-1} mbar, -25°C). Then dried samples were digested with papain (240 µl of papain, 9.76 ml of papain buffer 1x) overnight in a heat bath at 70° C.

Proteoglycan content

Quantifications of the sulfated-glycosaminoglycan content were made using a Dimethylmethylene Blue assay. After dilution with distilled water, digested samples were put in a 96-well ELISA microplate (U-bottom, Microton). 250µl of dying agent called Dimethylmethylene Blue (16mg of 1-9 dimethylmethylene blue, 2g of sodium formate, 2ml of formic acid, 1 liter of distilled water). Absorbance measures were taken at 620 nanometers.

A standard curve was prepared with chondroitin sulfate C from shark cartilage (Sigma). For each measure, triplicates were done. The mean was used to measure the glycosaminoglycan wet weight of each sample and these values were indicators of the proteoglycan content (Zhang et al, 2014).

Collagen content

To assess the collagen content, Hydroxyproline (OH-Pro) assays were conducted. These assays are an adaptation especially of the method coming from Bergman and Loxley (1963). 100µl of papain digested samples were mixed with 9M hydrochloric acid and were incubated overnight at 110°C in a sand bath. Then, hydrolysates were dried thanks to a SpeedVac Savant SC110A (Thermo Scientific) during 5 hours.

After dilution of the dried samples in 10ml of distilled water, 1ml of the solution was added in glass hydrolysis tubes with several other products: 1ml of diluent (100ml of propan-2-ol and 50ml of distilled water) and 1ml of oxidant (0.42g of chloramine T, 5ml of distilled water and 25ml of stock buffer made of sodium acetate, trisodium citrate.2H₂O, citric acid, propan-2-ol and distilled water). After a short incubation, 1ml of color reagent (3g DMBA, 25ml propan-2-ol, 4.5ml perchloric acid) was added. Samples were heated in a water bath at 70°C for 20 minutes. Hydroxyproline residues were oxidized by chloramine T and coupled with dimethylaminobenzaldehyde.

After an incubation at room temperature, absorbance (between yellow and pink) was harvested at 550 nanometers in a 96-well ELISA microplate (U-bottom, Microton). The standard curve was established with Trans-4-L-hydroxyproline (Sigma).

For each sample, triplicates were done. The mean was used to measure the hydroxyproline content of each sample. Then, collagen content in wet weight was calculated considering the fact that collagen is made of 14% of this residue (Zhang et al, 2014).

Water content

Each sample was weighted before and after freeze drying to assess the water content in %.

Quantitative real-time polymerase chain reaction

This technique was used to compare the effects of CS on proteoglycan and collagen contents at proteic and genic levels. This assessment could give insights about the way CS act on the extracellular matrix.

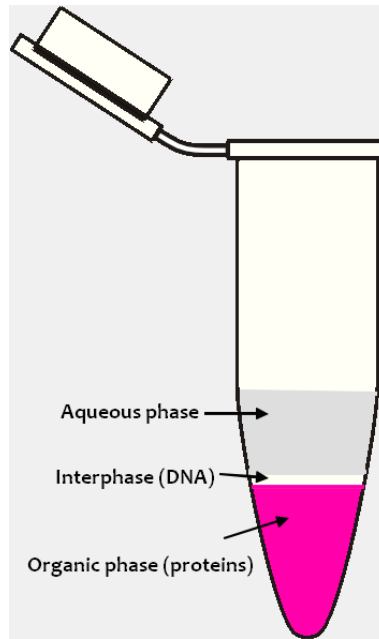


Figure 21: RNA extraction. The picture represents the separation into three phases of the sample thanks to centrifugation. The pink phase contains proteins (the color is due to the TRI-reagent solution). The white phase contains DNA. The colorless phase contains RNA.

Target gene	Orientation	Oligonucleotide sequence	Product size (bp)
Acan	Forward Reverse	5' CAGAGGAGCCATCCACACTT 3' 5' CAGAGGGCAACCTGTCAACT 3'	202
Col1a1	Forward Reverse	5' CCTGGTGACAAGGGTGAAAC 3' 5' CTTTAGCACCAGGCTGTCCAT 3'	378
Col2a1	Forward Reverse	5' GGCTCTCAGAACATCACCTACC 3' 5' GTACTCAATCACAGTCTCGCC 3'	204

Figure 22: Primer sequences used for Real-time PCR. First column: target gene; second column: orientation of the primer; third column: oligonucleotide sequence; fourth column: annealing product size in base pairs.

To quantify gene expressions, RNA extraction was performed (Vonk et al, 2010). After papain digestion, samples were used to isolate total RNA according to the protocol given by Sigma-aldrich. 1ml of a TRI-reagent solution (Sigma) was added to the samples and they were incubated 5 minutes at room temperature. Then they were centrifuged during 10 minutes (12000G, 4°C). The supernatant was transferred to a fresh tube and 200µl of chloroform were added. Samples were mixed and the resulting mixtures were incubated during 15 minutes at room temperature, and centrifuged during 15 minutes (12000G, 4°C). Centrifugation enabled to separate the mixture into three phases, including a colorless one containing RNA (**Figure 21**). This aqueous phase was transferred to a new tube and 500µl of isopropanol were added. After 1 hour of incubation at -80°C and a centrifugation of 15 minutes (12000G, 4°C), the supernatant was eliminated because pellets contained RNA. Pellets were centrifuged with 1ml of alcohol 70% during 5 minutes (7500G, room temperature), dried and suspended in 30µl of water. RNA quantity was assessed using a Nanodrop 2000c (Isogen Life Sciences).

Total RNA was reverse transcribed using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). After mixture of 5x Supermix (containing RNase inhibitor, dNTPs, primers, MgCl₂ and stabilizers) with Reverse Transcriptase, Nuclease-Free water and diluted RNA, samples were incubated in a thermal cycler using the following protocol: 5 minutes at 25°C (priming), 30 minutes at 42°C (reverse transcription) and 5 minutes at 85°C (enzyme inactivation). Then cDNA was stored at -20°C.

Targeted genes were Col2A1, Col1A1 and Acan. Their cDNA sequences were found on the website Ensembl. Specific primers were designed thanks to several tools: Primer 3, Net Primer and Geneious 8 (**Figure 22**).

Real-time PCR was realized thanks to the FastStart Universal SYBR Green Master (Rox) (Roches diagnostics) in an Eco Real-time PCR system (Illumina). cDNA samples were diluted 10 times in RNase-Free water. Mixtures for each genes (and for RNase-Free water, the negative control) were prepared in a 10µl total volume with 5µl SYBRGreen mix, 1.4µl RNase-Free water, 0.8µl forward primer (10mM), 0.8µl reverse primer (10mM) and 2µl of diluted cDNA. They were putted in a 48-well plate, centrifuged during 1 minute (1000rpm) and incubated in the Real-time PCR system using the following protocol: 3 minutes at 95°C (polymerase activation), 40 cycles in which one cycle included 30 seconds at 95°C (denaturation), 30 seconds at 50°C (annealing) and 30 seconds at 72°C (extension of primers), and then 30 seconds at 95°C, 15 seconds at 55°C and 15 seconds at 95° (final cycle).

Beta actin gene from *Ovis aries* was used as housekeeping gene and the gene expression levels were normalized compared to it. Results were analyzed using the softwares GeNorm and Ecostudy version 5.0.

3.4.2. HA dosage and MMPs activity

The techniques described in the first part to perform HA dosage and MMPs activity assessments were also used in this part.

3.4.3. HA fragment size

Large gel electrophoresis were performed to have an idea of the HA fragments sizes distribution pattern in the synovial fluid of one sheep before and after injections. The

objective was to assess whether the technique was feasible using very viscous ovine synovial fluid and to see if it would be an interesting outcome in the future.

To prepare the gel (agarose 5%), 198g of water were mixed with 1.1g of agarose. The solution was heated, cooled and water was added to come back to the initial weight of water. 22ml of TAE buffer (Tris base, acetic acid and EDTA) were added in the solution. The gel was poured into electrophoresis apparatus and polymerized during 3 hours.

Wells of the gel were filled in with equalized amount of HA (7 μ g). The final volume was 25 μ l and synovial fluid was mixed with water to have amount of 7 μ g of HA. Three standards were used: 400000 Daltons, 1600000 Daltons and 2500000 Daltons.

3.5. Statistical analysis

Data were collected in Microsoft Excel and analyses were performed using R Studio. Kolmogorov-Smirnov tests were used to examine the normality of data. Due to the non-normality of most of data, we chose to apply only non-parametric tests. Mann-Whitney-Wilcoxon tests were performed to assess the significance of the results. Regression linear models were performed to assess potential correlation between age and MMPs activities. A p-value less than 0.05 was considered as statistically significant.

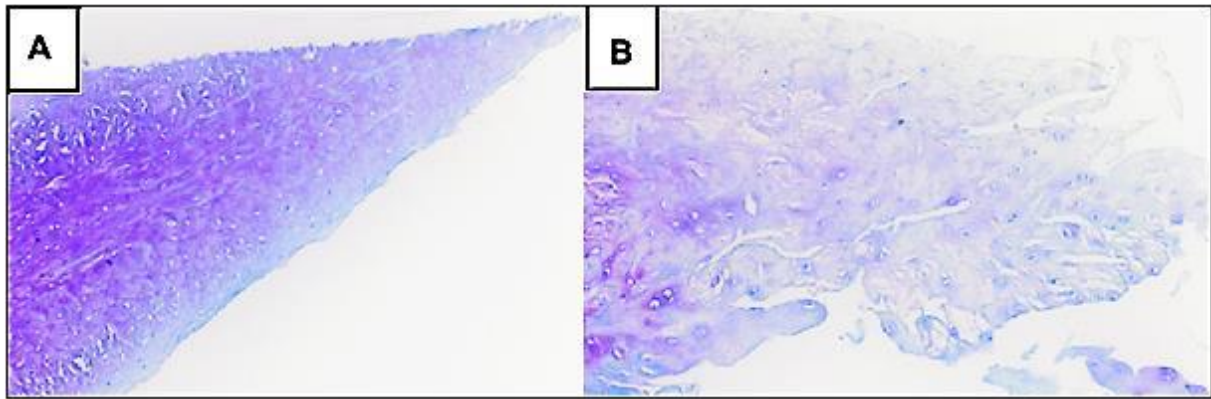


Figure 23: Photomicrographs of sheep menisci histological slices. A: healthy young meniscus, with a good extracellular matrix staining, a good architecture and no cellular proliferation. 2: older meniscus, which has lost its staining and its architecture. The staining was Toluidine blue and it allowed to highlight glycosaminoglycan and proteoglycans contents.

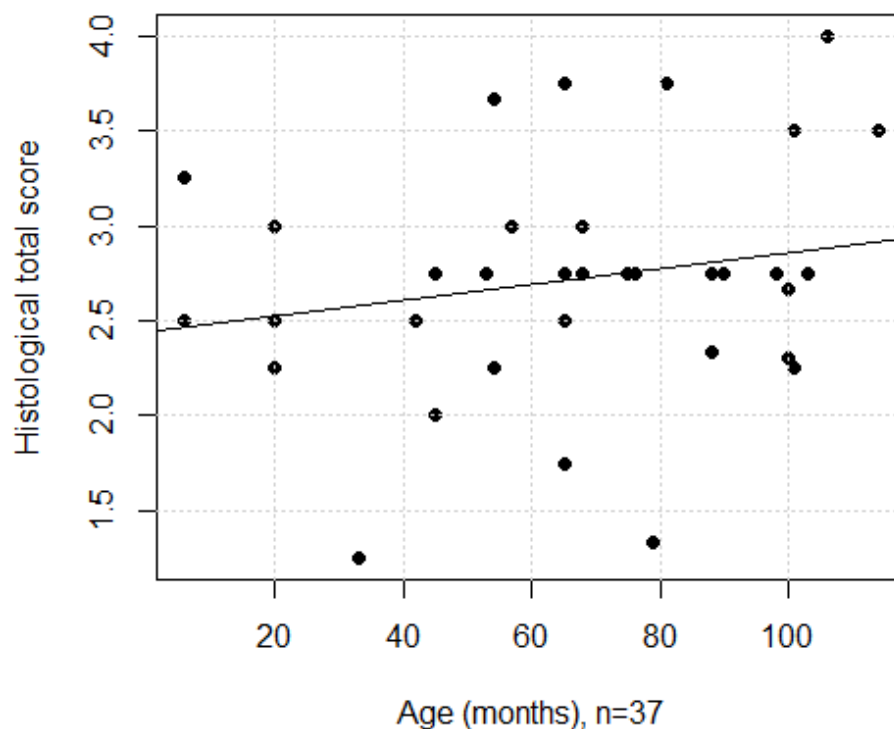


Figure 24: Correlation between histological total scores and the age of the animals. The x axis indicates the age in months, and the y axis indicates the histological total OARSI score. This total mean score has been obtained by pooling scores of lateral and medial menisci, and scores of left and right limbs. It was possible because no significant differences were observed between lateral and medial menisci ($p = 0.250$) and between left and right limbs ($p=0.189$). An analysis of the correlation by a linear regression model showed that the slope was not significant ($p = 0.201$). It highlights a lack of significance of the correlation between the two variables.

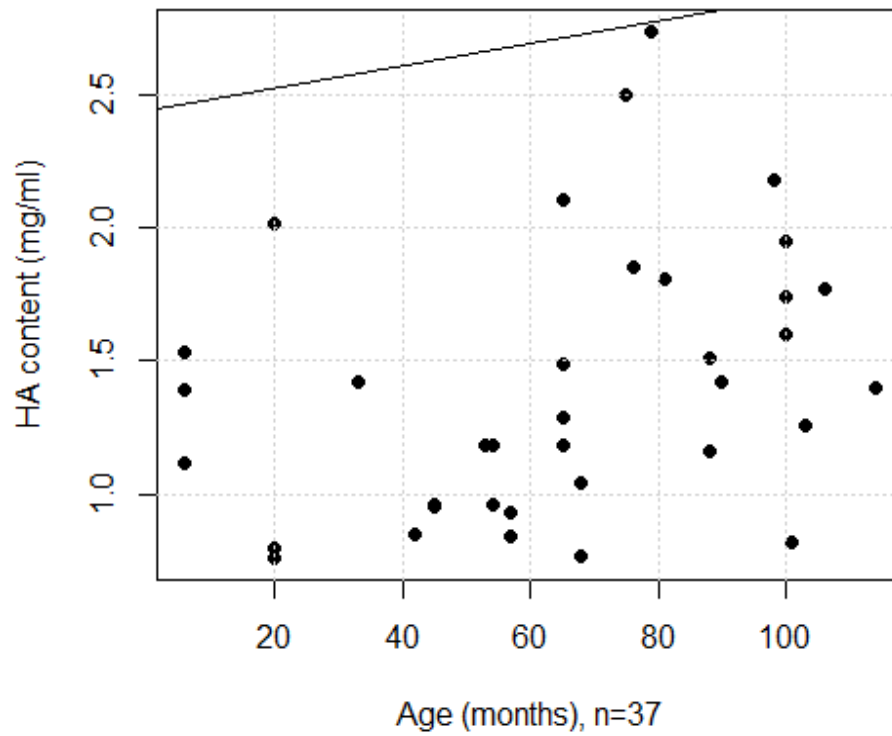


Figure 25: Correlation between HA content and the age of animals. The x axis indicates the age in months, and the y axis indicates the HA concentration in mg/ml. A total mean content for each sheep has been obtained by pooling values from left and right limbs. It was possible because no significant differences were observed between left and right limbs ($p = 0.937$). An analysis of the correlation by a linear regression model showed that the slope was not significant ($p = 0.104$). There were no correlation between the two variables.

IV. RESULTS

1. First part: preliminary prevalence study

37 crossed Texel ewes were used for the study. The minimum age in the population was 6 months, the mean age was 65 months (5 years and 5 months) and the maximum age was 114 months (9 years and 6 months). 15 sheep were younger than the mean age and 22 sheep were older than 5 years and 5 months. Since sheep retired from breeding are rarely older than 11 years, our population covers the whole possible range of ages of research sheep.

1.1. Histology of meniscus

On the 148 histological samples, six could not be analyzed because of artifacts on the slices. For each sheep, middle parts of the lateral and medial menisci of the left and right knees were examined. Therefore, four scores were obtained. No statistical differences were observed between scores of each meniscus (medial *versus* lateral) of each limb (left: $p = 0.205$; right: $p = 0.300$) and between mean scores of each limb (left *versus* right: $p = 0.189$). Mean total scores per sheep were therefore calculated from the sum of the four scores.

Figure 23 shows examples of histological slices scored during the study. Picture A is the photomicrograph of a healthy young meniscus. It has a good glycosaminoglycan content and a good architecture. It is not the case for Picture B which shows an older meniscus. It has lost its staining and it is submitted to severe alterations because the normal triangle shape disappeared.

Figure 24 is a statistical analysis of the correlation between total histological scores and ageing. The linear regression model showed no significant correlation between the two variables ($p = 0.201$), indicating that age does not affect structure of menisci in our research population, i.e. sheep between 6 months and 9 and a half years old without clinical signs of OA.

1.2. Synovial fluid analysis

1.2.1. HA content

Due to the difficulty to sample synovial fluid, it could be collected bilaterally in 27 sheep and unilaterally in 10 sheep. Statistical tests showed no significant differences between HA content from left and right limbs in sheep where samples had been obtained bilaterally ($p = 0.937$). Therefore, mean values were calculated based on the sum of the HA contents obtained from left and right limbs, also for animals with unilateral samplings.

Figure 25 is a statistical analysis of the correlation between the HA content and ageing. The linear regression model showed no significant correlation between the two variables ($p = 0.104$).

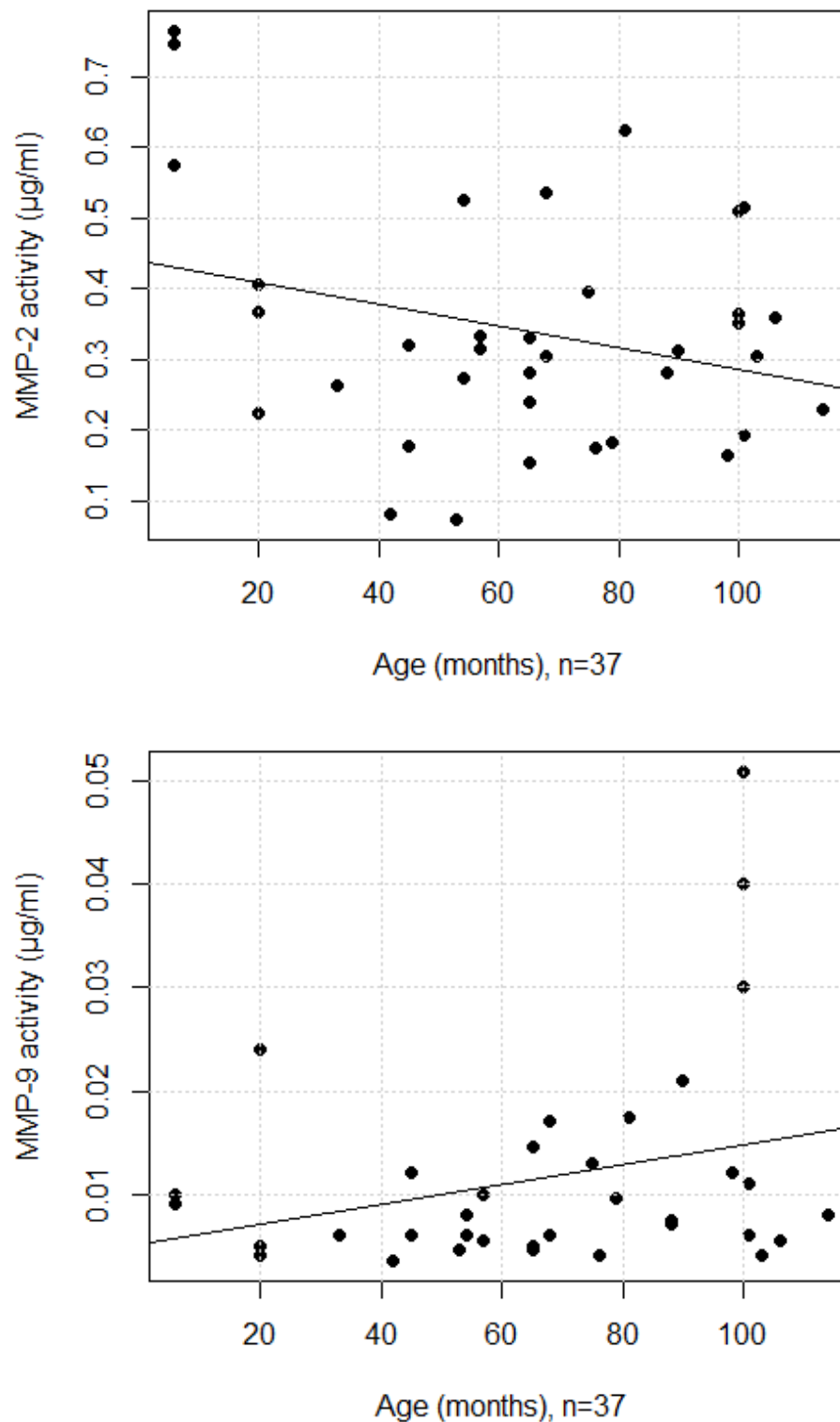


Figure 26: Correlation between MMP-2 and -9 activities and the age of animals. X axis indicates the age in months while y axis indicates the MMP activity in $\mu\text{g/ml}$. Total mean activities for each sheep have been obtained by pooling values from left and right limbs. It was possible because no significant differences were observed between left and right limbs ($p = 0.933$ for MMP-2 and $p = 0.371$ for MMP-9). First graph: an analysis of the correlation by a linear regression model showed that the slope was not significant ($p = 0.085$). Second graph: the analysis showed that the slope was not significant ($p = 0.082$). Although p-values were close to the significance, there were no impact of ageing on MMP-2 and -9 activities in the synovial fluid in this population of sheep.

1.2.2. MMP-2 and MMP-9 activities

On 64 synovial fluid samples, 10 samples for MMP-2 and 15 samples for MMP-9 could not be analyzed due to a lack of synovial fluid. Statistical tests showed no significant differences between activities from left limbs and activities from right limbs ($p = 0.933$ for MMP-2; $p = 0.371$ for MMP-9). Therefore, mean values were calculated based on the activities obtained in both limbs.

Figure 26 is a statistical analysis of the correlation between MMP-2 and MMP-9 activities and ageing. Linear regression models showed no significant correlation between age and MMP-2 ($p = 0.085$) and MMP-9 ($p = 0.082$).

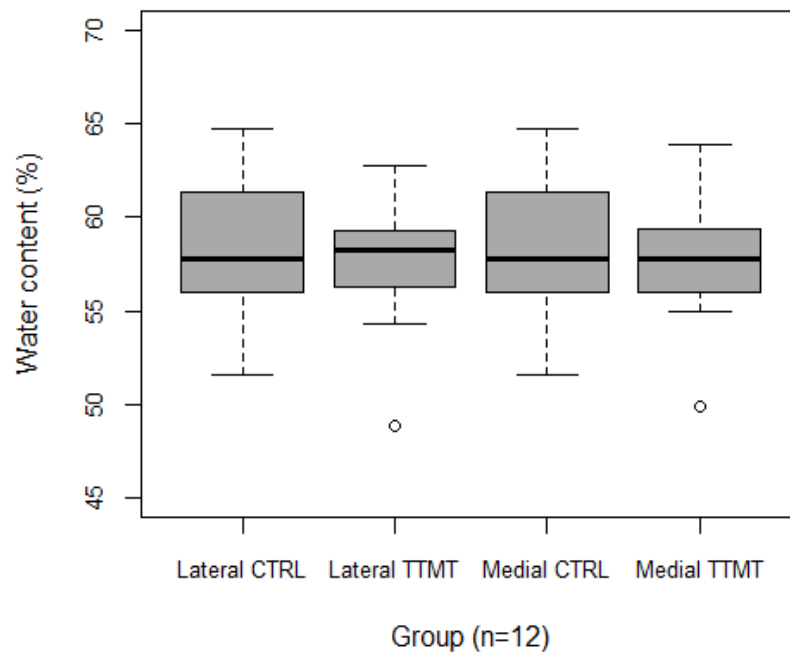


Figure 27: Boxplots showing the effect of 25mg triamcinolone hexacetonie on the water content of lateral and medial menisci in sheep. X axis indicates the group: Lateral CTRL = lateral menisci submitted to saline injections; Lateral TTMT = lateral menisci submitted to TH injections; Medial CTRL = medial menisci submitted to saline injections; Medial TTMT = medial menisci submitted to TH injections. Y axis indicates the water content in %. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and circles represent extreme values. Statistical significance was analyzed by Wilcoxon-Mann-Withney tests.

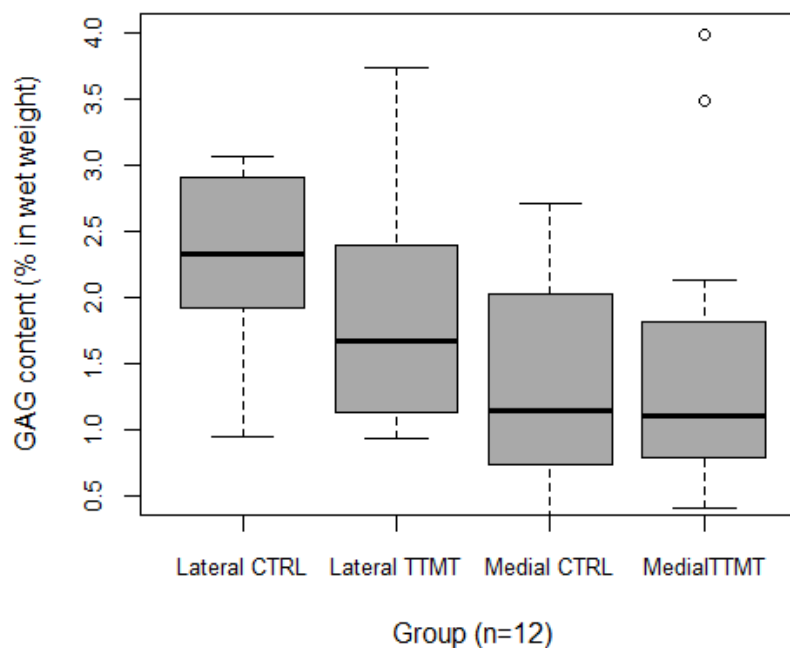
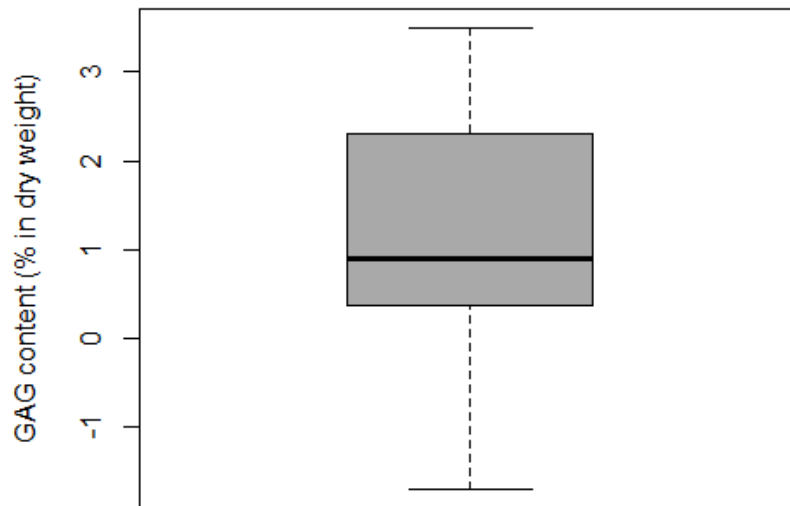


Figure 28: Boxplots showing the effect of 25mg triamcinolone hexacetonide on the GAG content of lateral and medial menisci in sheep. X axis indicates the group: Lateral CTRL = lateral menisci submitted to saline injections; Lateral TTMT = lateral menisci submitted to TH injections; Medial CTRL = medial menisci submitted to saline injections; Medial TTMT = medial menisci submitted to CS injections. Y axis indicates the GAG content in % of wet weight. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and circles represent extreme values. Statistical significance was analyzed by Wilcoxon-Mann-Withney tests.



LateralTTMT-MedialTTMT (n=12)

Figure 29: Boxplot showing the significant difference of treatment response between lateral and medial menisci GAG contents. X axis indicates that values represented on this graph are the difference between LateralTTMT (i.e. lateral menisci submitted to CS injections) and MedialTTMT (i.e. medial menisci submitted to TH injections). Y axis indicates the GAG content in % of dry weight. The box represents the distribution of 50% of the values and the black bold line indicate the median value. Statistical significance was analyzed by a Wilcoxon-Mann-Withney test and was positive for this boxplot.

2. Second part: study of the effects of triamcinolone hexacetonide

15 ewes were used for the study. The minimum age in the population was 24 months (2 years), the mean age was 80 months (6 years and 8 months) and the maximum age was 130 months (10 years and 10 months). 6 sheep were younger than the mean age and 9 sheep were older than 6 years and 8 months.

2.1. Biochemistry of meniscus

A total of 60 menisci were sampled. On the 60, 9 could not be analyzed due to problems with samples preservation.

2.1.1. Water content

Figure 27 indicates the effect of TH on lateral and medial meniscal water contents. The two first boxplots represent lateral menisci of control and treatment groups while the two others represent medial menisci of both groups. Lateral menisci of the control group have median value of 57.74% and lateral menisci of the treatment group median value of 58.26%. Medial menisci of the control group have a median value of 57.75% and medial menisci of the treatment group have a median value of 57.77%. No trends appear on this graph.

Statistical analysis confirmed no significant differences between lateral menisci of control and treatment groups ($p = 0.301$) and between medial menisci of control and treatment groups ($p = 0.477$). Response to saline injections and TH injections were not statistically different between medial and lateral menisci ($p = 0.136$ and 0.693 respectively).

2.1.2. GAG content

Figure 28 indicates the effect of TH on lateral and medial meniscal GAG contents in wet weight. The two first boxplots represent lateral menisci of control and treatment groups, while the two other boxplots represent medial menisci of both groups. Lateral menisci of the control group have a median value of 2.33% and lateral menisci of the treatment group have a median value of 1.67%. Medial menisci of the control group have a median value of 1.15% and medial menisci of the treatment group have a median value of 1.1%. The visible trend is a decrease in the GAG content after TH injections.

However, statistical analysis showed no significant differences between lateral menisci of control and treatment groups ($p = 0.176$) and between medial menisci of control and treatment groups ($p = 0.850$). The response to saline injections and to TH injections were not statistically different between medial and lateral menisci ($p = 0.175$ and 0.278 respectively).

Statistical comparisons was also made on GAG content in dry weight and showed a significant difference ($p = 0.049$) between the treatment response of lateral and medial menisci. This difference is represented by **Figure 29**. Lateral menisci seem to be more disrupted by CS treatment than medial menisci.

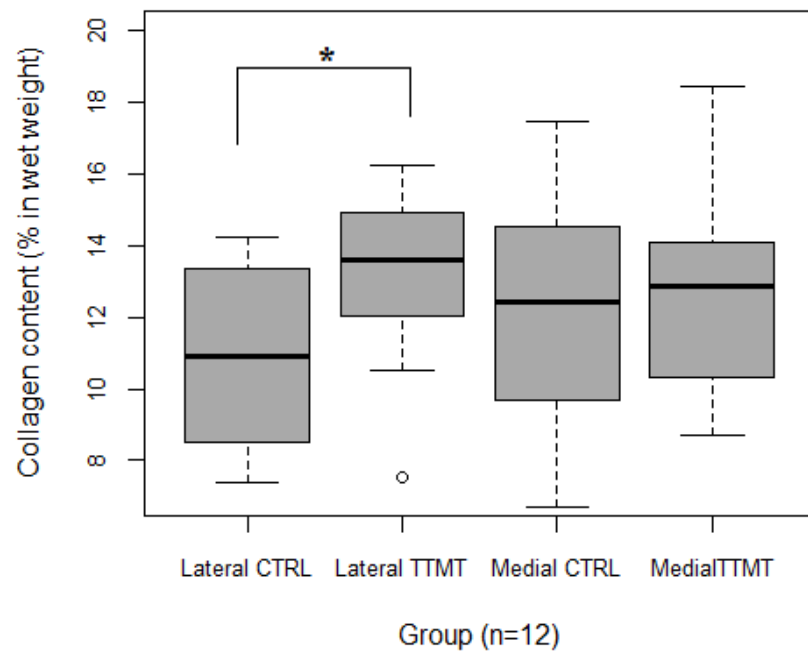


Figure 30: Boxplots showing the effect of 25mg triamcinolone hexacetonide on the collagen content of lateral and medial menisci in sheep. X axis indicates the group: Lateral CTRL = lateral menisci submitted to saline injections; Lateral TTMT = lateral menisci submitted to TH injections; Medial CTRL = medial menisci submitted to saline injections; Medial TTMT = medial menisci submitted to TH injections. Y axis represents the collagen content in % of wet weight. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and the circle represents an extreme value. Statistical significance was analyzed by Wilcoxon-Mann-Withney tests (*: $p < 0.05$).

2.1.3. Collagen content

Figure 30 indicates the effect of TH on lateral and medial meniscal collagen contents in wet weight. The two first boxplots represent lateral menisci of control and treatment groups, while the two other boxplots represent medial menisci of both groups.

Lateral menisci of the control group have a median value of 10.93% and lateral menisci of the treatment group have a median value of 13.57%. Medial menisci of the control group have a median value of 12.42% and medial menisci of the treatment group have a median value of 12.87%. The visible trend is an increase in the collagen content after treatment.

Statistical analysis showed a significant difference between lateral menisci of the control group and treatment groups ($p = 0.012$). This significance was not present between medial menisci of the control and treatment groups ($p = 0.909$). The response to saline injections was not statistically different between medial and lateral menisci ($p = 0.831$). The treatment response was not statistically different between medial and lateral menisci ($p = 0.320$).

2.1.4. Study of extracellular matrix genes expression

A RT-qPCR was performed to quantify the transcriptional expression of three extracellular matrix genes: aggrecan (Acan), type-II collagen (Col2A1) and type-I collagen (Col1A1). It was interesting to assess whether CS injections could cause any change at a transcriptional level or not. Results of this experiment could not be analyzed. Despite we found a good housekeeping gene (β -actin gene), it turned out that RNA extraction from menisci was very difficult. The TRI-reagent protocol is made to extract RNA from suspension cells or solutions. We therefore tried to dissolve menisci into papain solution but we found, after qPCR analysis that papain impaired RNA and impeded to remove genomic DNA contaminations. We also tried to extract RNA from meniscal tissues cooled in liquid nitrogen and broke into small pieces but it did not work. The RNA purity was not sufficient, even after purification technique or DNase treatment. At present, we did not find a way to dissolve menisci easily, and there was no tissue homogenizer in the lab. Besides, the design of primers was also very difficult because of the high GC content in the three extracellular matrix genes sequences. An electrophoresis highlighted that primers made a lot of non-planned dimers. Due to the lack of time, it was not possible to edit them or to test other melting temperatures.

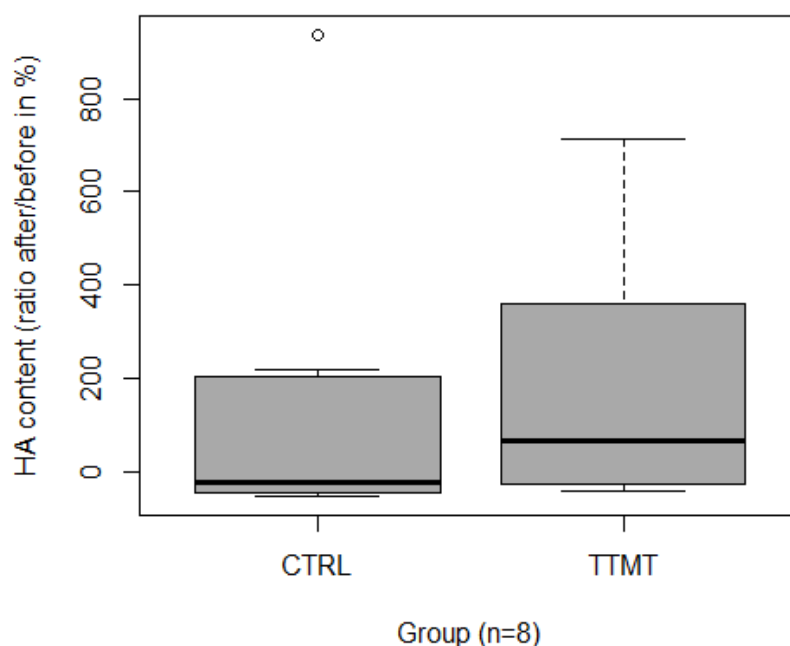


Figure 31: Boxplots showing the effect of 25mg triamcinolone hexacetonide on the HA content in the synovial fluid. X axis indicates the group: CTRL = synovial fluids from knees submitted to saline injections; TTMT = synovial fluids from knees submitted to TH injections. Y axis represents the HA content. It is expressed in ratio “after injection/before injection” to remove the calibration effect. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and the circle represents an extreme value. Statistical significance was analyzed by a Wilcoxon-Mann-Withney test.

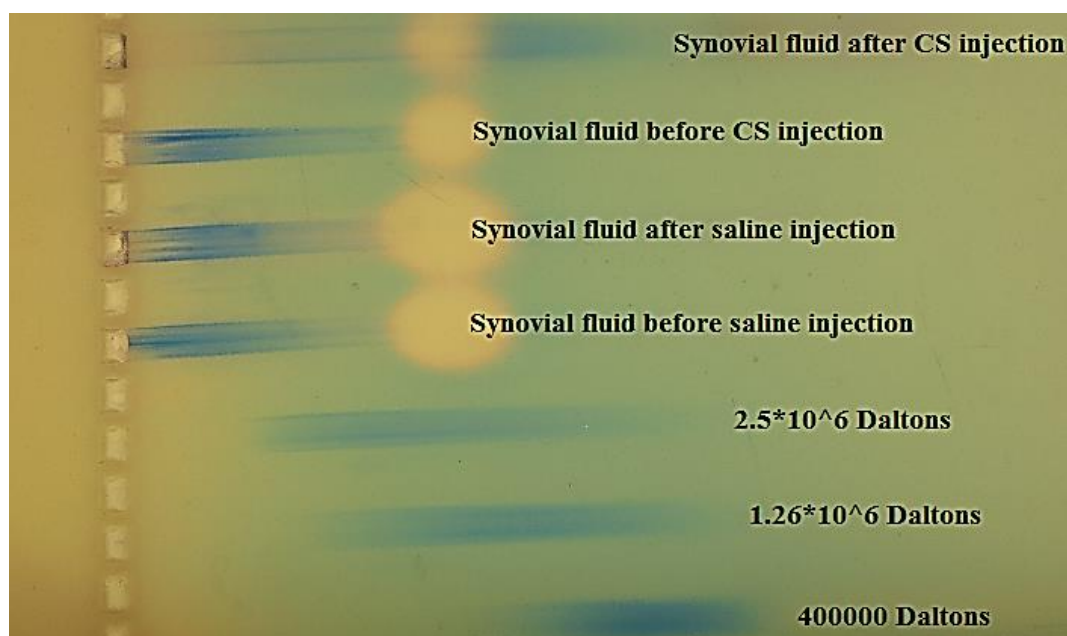


Figure 32: Electrophoresis gel showing the effect of 25mg triamcinolone hexacetonide on the HA fragments size in the synovial fluid. The amount of loaded HA was 7μg (in a final volume of 25μl). Three standards were put in the gels: 400000 Daltons, 1600000 Daltons and 2500000 Daltons. The gel contains 4 samples of one sheep: before and after saline injection in one knee, before and after TH injection in the contralateral knee.

2.2. Synovial fluid analysis

2.2.1. HA content

Due to the difficulty to sample synovial fluid and because of its scarcity in some knee joints, we only could analyze results of 8 sheep on 15.

Figure 31 indicates the effect of TH on the HA content in the ovine synovial fluid. The first boxplot represents synovial fluids from knees submitted to saline injections while the second boxplot represents synovial fluids from knees submitted to CS injections. The control group has a median ratio of 0.78 while the treatment group has a median value of 1.64. The visible trend is an increase in the HA content after treatment.

However, statistical analysis showed a non-significant difference between control and treatment groups ($p = 0.547$).

2.2.2. HA fragments size

Figure 32 shows that the HA fragments size distribution patterns in the synovial fluid before saline injection, after saline injection and before TH injection are different from the pattern seen in the synovial fluid after TH injection. HA fragments seem to be smaller after treatment than after saline injection. Another gel was made and did not show that clear differences. The small amount of data did not allow to perform statistical analysis but showed the feasibility of the technique on ovine synovial fluid.

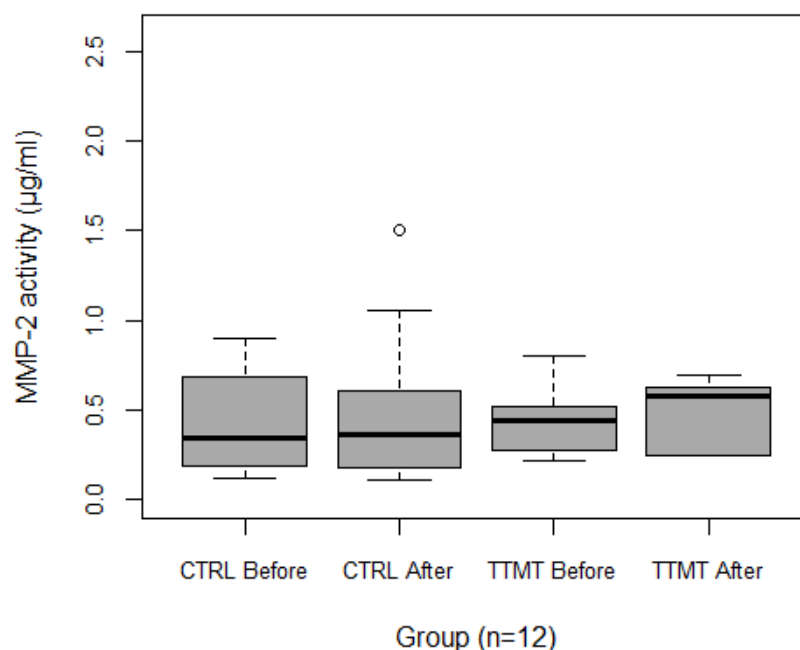


Figure 33: Boxplots showing the effect of 25mg triamcinolone hexacetonide on MMP-2 potential activity in the ovine synovial fluid. X axis indicates the group: CTRL Before = synovial fluids from knees of the control group before saline injections; CTRL After = synovial fluids from knees of the control group after saline injections; TTMT Before = synovial fluids from knees of the treatment group before TH injections; TTMT After = synovial fluids from knees of the treatment group after TH injections. Y axis indicates the MMP-2 potential activity in $\mu\text{g/ml}$. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and circles represent extreme values. Statistical significance was analyzed by Wilcoxon-Mann-Withney tests.

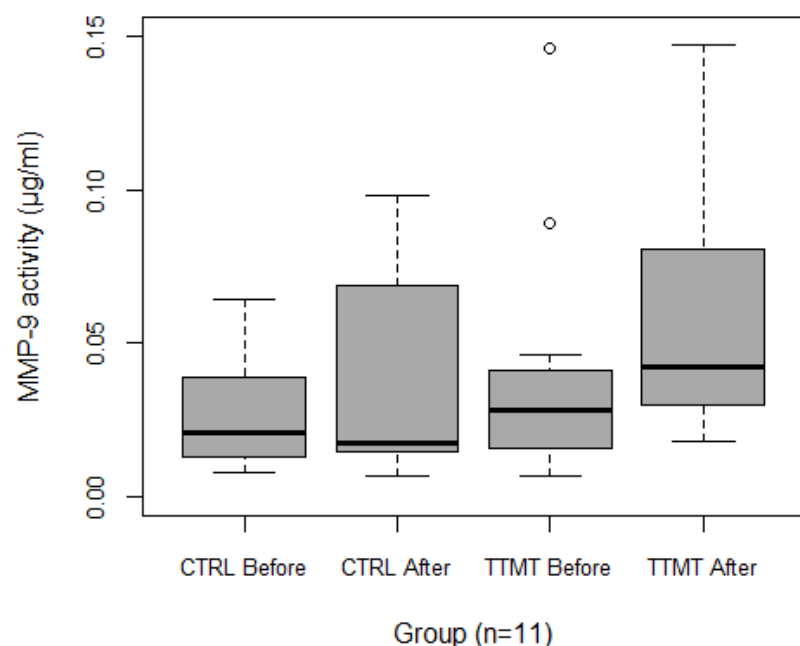


Figure 34: Boxplots showing the effect of 25mg triamcinolone hexacetonide on MMP-9 potential activity in the ovine synovial fluid. X axis indicates the group: CTRL Before = synovial fluids from knees of the control group before saline injections; CTRL After = synovial fluids from knees of the control group after saline injections; TTMT Before = synovial fluids from knees of the treatment group before TH injections; TTMT After = synovial fluids from knees of the treatment group after TH injections. Y axis indicates the MMP-9 activity in $\mu\text{g/ml}$. Boxes represent the distribution of 50% of the values, black bold lines indicate median values and circles represent extreme values. Statistical significance was analyzed by Wilcoxon-Mann-Withney tests.

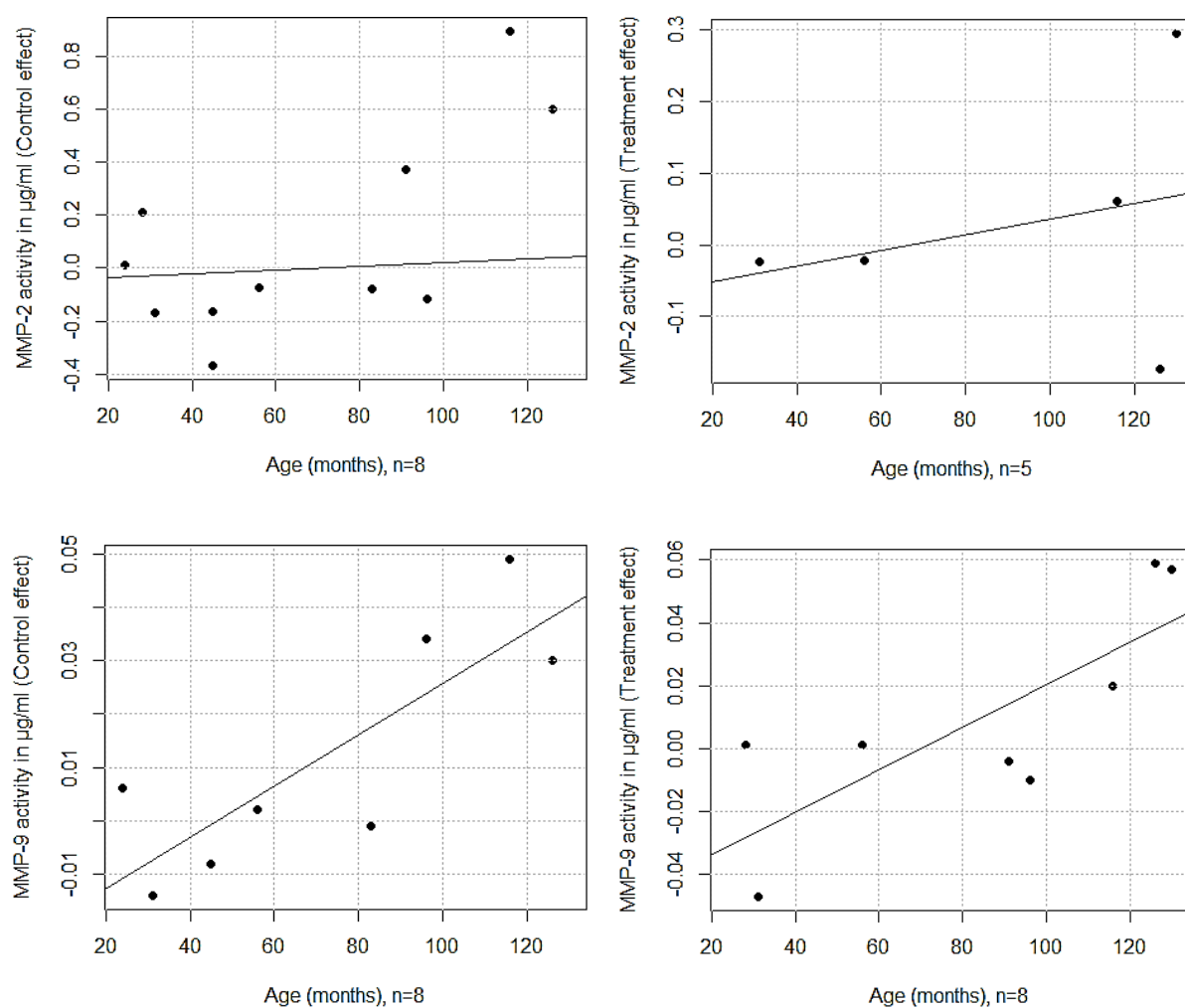


Figure 35: Correlation between MMP-2 and -9 potential activities in the synovial fluid and the age of animals. X axis indicate the age in months while y axis indicate the MMP potential activity in $\mu\text{g/ml}$. **LEFT:** an analysis of the correlation by linear regression models showed that slopes were significant ($p = 0.023$ for MMP-2 and 0.012 for MMP-9). **RIGHT:** For MMP-2, the potential activity in treatment group was not influenced by age ($p = 0.638$) while it was significantly influenced by age for MMP-9 ($p = 0.021$).

2.2.3. MMP-2 and MMP-9 activities

Figure 33 indicates the effect of TH on MMP-2 potential activity in the ovine synovial fluid. The two first boxplots represent synovial fluids from knees of the control group before and after saline injections while the two others represent synovial fluids from knees of the treatment group before and after TH injections. The control group has a median activity of 0.34µg/ml before injections and a median activity of 0.36µg/ml after injections. The treatment group has a median activity of 0.44µg/ml before injections and a median activity of 0.58µg/ml after injections. The visible trend is an increase in the activity after treatment.

However, statistical analysis showed no significant differences between the control group before and after injections ($p = 0.700$) and between the treatment group before and after injections ($p = 0.688$). Activities in the two groups were not statistically different neither before ($p = 0.765$) nor after injections ($p = 0.625$).

Figure 34 indicates the effect of TH on MMP-9 potential activity in the ovine synovial fluid. The boxplots represent the same groups as in the previous figure. The control group has a median activity of 0.02µg/ml before injections and a median value of 0.02µg/ml after injections. The treatment group has a median activity of 0.03µg/ml before injections and a median value of 0.04µg/ml after injections. The visible trend is an increase in the activity after treatment.

However, statistical analysis showed no significant differences between the control group before and after injections ($p = 0.313$) and between the treatment group before and after injections ($p = 0.547$). Activities in the two groups were not statistically different neither before ($p = 0.831$) nor after injections ($p = 1$).

As the impact of ageing on MMPs activities was almost significant in the preliminary study ($p = 0.085$ for MMP-2 and 0.082 for MMP-9), we decided as a precaution to assess if ageing had an impact on MMPs activities in this population of sheep. **Figure 35** showed a significant correlation between ageing and MMP-2 activities in the control group ($p = 0.024$) and between ageing and MMP-9 activities in both groups ($p = 0.012$ in the control group and 0.020 in the treatment group).

V. DISCUSSION

Intra-articular CS injections have been widely used for decades to treat knee OA (Maricar et al, 2013). However, there is a lack of accurate information about the effects these drugs could have on different components of the joint such as articular cartilage, menisci and synovial fluid (Ishikawa et al, 1981; Jüni et al, 2015; Vandeweerdt et al, 2015). This master thesis studied TH effects on sheep knee joint, as the ovine model is commonly used in OA research (Little et al, 2010), and focused on menisci and synovial fluid characteristics (HA content and MMPs activity). TH was used because previous report evidenced it could be an intra-articular steroid of choice (Cheng et al, 2012).

To ensure that potential CS effects observed in the study were well due to the drugs and not to other variables, we chose only female animals with no orthopedic diseases (selection based on clinical evaluation and medical imaging) and submitted to standardized diet. Although age-related changes in the articular cartilage at macroscopic, histological and biochemical levels have already been documented in sheep (Vandeweerdt et al, 2013; Matagne et al, 2014; Pirson et al, 2014), they have not been studied in menisci and synovial fluid. Therefore, the first part of this work consisted of a preliminary cross-sectional study trying to complement current knowledge about the impact of ageing on meniscal cartilage and synovial fluid.

The preliminary study brought some trends, but statistical analysis showed that all trends were not significant. In oldest animals, we observed by histological scoring that the architecture of the menisci seemed to be altered. Over years, menisci showed signs of extracellular matrix loss, as evidenced by a decrease in the Toluidine blue staining which highlighted proteoglycan and glycosaminoglycan contents. The increase in biomechanical strains with increasing years could induce meniscal lesions (Gepstein et al, 2002). No trend appeared in the evolution of HA content with years. MMP-2 potential activity decreased with years while MMP-9 potential activity increased. We suggest, as other authors, that the increase in MMP-9 activity could be due to synovial cells stimulation either by aged cartilage fragments in contact with the synovium or by an oxidative stress augmentation in the environment of synovial cells (Shane Anderson et Loeser, 2010; Berenbaum et al, 2013).

As results obtained for MMP-2 and MMP-9 were not far from significance, we had to remain careful when we analyzed CS effects on these parameters.

In the second part of this thesis, we focused on the potential CS effects (i) on the biochemistry of menisci by assessing water, proteoglycan/glycosaminoglycans and collagen contents, and (ii) on the inflammatory state of the synovial fluid by assessing HA content, HA fragments size and MMP-2 and -9 potential activities.

Biochemistry of meniscus

Menisci play an essential role in the biomechanics of the joint. Their mechanical properties depend on the biochemical composition of the extracellular matrix, i.e. collagen, water and proteoglycan contents. Collagenous components play a role in the resistance to tensile forces developed in the outer meniscal zones. Water and proteoglycans are involved in the shock

absorption and in the resistance to inner compressive forces (McDevitt et Webber, 1990; Gruber et al, 2008; Brindle et al, 2011; Vanderploeg et al, 2012).

Biochemical assessments in the meniscus after CS injections are relevant because the extracellular matrix can be disrupted in OA. In case of disease, collagen content appear to decrease leading to a loss of mechanical resistance (Sun et al, 2012; Levillain et al, 2015). Some studies reported a decrease of the content in proteoglycans, due to the activation of proteoglycan-degrading enzymes, followed by a recovery after few weeks thanks to meniscal regeneration (Adams et al, 1983; Herwig, 1984). Other studies showed an increase in the content due to a stimulation of the GAG synthesis (Ghosh et al, 1975; Videman et al, 1979; Hellio Le Graverand et al, 2001; Sun et al, 2012). These differences were reported to be due to the type of OA model.

The exact biochemical composition of menisci is still controversial. In man, studies highlighted that normal meniscus contained 70% of water, between 20 and 25% of collagen and small amounts of proteoglycans (< 2%) in wet weight (Herwig et al, 1984; Fithian et al, 1990). In porcine and bovine menisci, the GAG-fraction in dry weight appears to be slightly different: 8% of GAG in inner and middle meniscal zones and 2% in outer zones for pigs, and a maximum of 4% in bovine menisci (Nakano et al, 1997; Adam-Sanchez 2011).

Several reasons could explain the differences in the percentages we obtained with the other studies. Firstly, although the water content seems to be similar in the whole meniscus (Fox et al, 2012), studies revealed that the collagen and proteoglycan contents depended on the cross-sectional localization (Zhang et al, 2010; Fuller et al, 2012; Sun et al, 2012; Vanderploeg et al, 2012; Moyer et al, 2013). Our study only assessed the contents in a meniscal cross-section, without any differentiation of the zone. Secondly, these differences could be due to a potential interspecies variation (McDevitt et Webber, 1990). Thirdly, the low values obtained in collagen content could be due to the fact that the detection was made thanks to a technique adapted for articular cartilage assessment. The standard came from shark cartilage. It was the only standard available but it could not be the best to assess collagen content in menisci. It could give underestimations of the content. Nevertheless, as all samples were submitted to the same standard, comparisons between control group and treatment group remain valid.

Effects of CS on the three components are poorly documented by peer reviewed publications.

Ishikawa et al., in rabbits, showed deleterious effects on proteoglycan and collagen contents after repeated injections of prednisolone acetate (Ishikawa et al, 1981). They found a decrease in the proteoglycan and collagen synthesis and a disruption of the interactions between these constituents. Yamashita et al., in dogs, showed a decrease in the extracellular matrix content and a disorganization of the collagen bundles after the intra-articular injection of a high dose of halopredone acetate (Yamashita et al, 1986). In our study, after the intra-articular injection of TH, water content remained stable and proteoglycan content had tendency to decrease. It was in agreement with the studies described above but statistical analysis revealed non-significant differences not allowing to draw conclusions.

In the current study, the collagen content had tendency to increase after treatment. The results were significant only in lateral menisci. This is in contradiction with the studies described

above, but these results have already been obtained in other studies. Sanchez-Adams et al. showed that the increase in collagen content could be explained by the fact that as the GAG-fraction is slightly disrupted, this could lead to a concomitant increase in collagen content in term of proportions (Sanchez-Adams et al, 2011). Besides, as the half-life of collagen is much longer than proteoglycan half-life (300 days *versus* 10 days), it is supposed that real significant changes after CS injections would appear first in proteoglycan content. Moreover, sheep were euthanized four weeks after CS injections, and even a total disruption of the collagen synthesis would cause only minor changes in hydroxyproline concentrations since the turnover is very slow (Behrens et al, 1975).

Our study showed that the TH had apparently no side effects on the biochemistry of the ovine meniscus. Besides, to our knowledge, this work is the first which differentiate lateral and medial menisci. In wet weight, no significant differences were observed between both menisci. However, dry weight basis analysis showed that the GAG content reacted significantly differently to treatment in lateral and medial menisci. Although it may seem surprising, a difference of significance between wet weight and dry weight has already been obtained in human medicine (Herwig et al, 1984). Moreover, as there were no significant effects of CS on the water content, it is possible that when water is removed, significant differences appear in dry weight.

The difference in response to treatment between lateral and medial menisci could be due to the fact that during knee flexion, the lateral meniscus transmits the totality of the load while the medial meniscus transmits only 50% (Walker et Hajek, 1972). This difference in biomechanical strains could induce different reactions of the extracellular matrix to CS treatment. It is consistent with our results because GAG content decreased more in lateral menisci than in medial menisci, although it was not significant.

A RT-qPCR was performed to highlight a potential difference of transcriptional expression of extracellular matrix genes (aggrecan, type-II collagen and type-I collagen) due to triamcinolone injections. However, results of this experiment could not be analyzed due to a lack of RNA purity and primer quality, despite the choice of a good housekeeping gene (β -actin gene) whose expression remained stable in the experiment.

Synovial fluid analysis

HA is a major component of the synovial fluid. It gives to the fluid its viscoelastic properties and play an important role in the shock absorption and lubrication of the joint (Plickert et al, 2013). Studies in dogs and rabbits showed that the concentration of this non-sulfated glycosaminoglycan decreased in case of OA, probably due to an alteration of its synthesis and accelerated metabolization (Liu et al, 2012; Plickert et al, 2013). Moreover, HA could be a biomarker allowing to monitor intra-articular treatments (Decker et al, 1959). It is therefore relevant to assess its content after TH injections.

The quantification of HA in synovial fluid is still imprecise. For example, in sheep, Fraser et al. showed a mean HA concentration of 0.5 mg/ml while Barton et al. showed more recently a concentration of 0.9 mg/ml (Fraser et al, 1993; Barton et al, 2013). Results vary according to the detection method, to the sample, to the joint and to the species (Fraser et al, 1993; Liu et

al, 2012). However, this inaccuracy has little effect on the comparison between the control group and the CS treatment group of the two ELISA-like microplates because we took a ratio “after injection/before injection” to remove the calibration effect.

After intra-articular CS injection, Decker et al. described no significant effects on the HA content (Decker et al, 1955). More recent studies in man and horses showed that the HA content tended to increase initially (Tulamo et al, 1991; Ronéus et al, 1993) and this increase was followed by a subsequent decrease (Pitsillides et al, 1994).

In analogy to foregoing results, we showed a non-significant increase in HA concentrations after treatment. This trend could be explained by potential changes in the turnover of HA, as it is the case in inflamed joints. Fraser et al. showed that the mean half-life of HA (0.5-1 day) decreased in case of inflammation (Fraser et al, 1993). The increase in HA content could be due to an irritation state caused by the TH injection, as it was the case in studies in horses. The irritation, together with a reduction of viscosity, had a stimulatory effect and led to an increase in HA synthesis by synovial cells (Ronéus et al, 1993; Soltés et al, 2005). Another explanation could be that CS reduced the synovial membrane permeability, leading to an increased HA concentration in the fluid (Tulamo et al, 1991; Ronéus et al, 1993).

As explained in the introduction, HA has different effects on the inflammation in function of its molecular weight (Petrey et de la Motte, 2014). Studies showed that the elasticity of the joint increased with increasing HA fragments size (Tamer, 2013) and that its molecular weight was decreased in inflamed joints (Plickert et al, 2013) and OA-associated synovitis (Band et al, 2015). Wang et al. showed that high molecular weight fragments down-regulated the expression of aggrecanases and MMPs, suggesting that these fragments had anti-inflammatory effect (Wang et al, 2006). Moreover, a recent review of the literature highlighted the fact that increased levels of low molecular weight HA have been found in inflammatory diseases like rheumatoid arthritis (Cyphert et al, 2015).

Although a study already showed that the average HA molecular weight ranged between 1 and 2.10^6 Daltons in the ovine synovial fluid (Adam et Ghosh, 2001), to our knowledge, our study is the first that tries to assess whether changes in the HA molecular weight occur after CS injections or not. On one of the two gels prepared, we observed a decrease in the molecular weight after triamcinolone injections. It could be due to a degradation by reactive oxygen species, catabolic enzymes, or fragments could be produced directly as shorter chains by different HA synthases (Halliwell, 1995; Usuki et al, 2008; Petrey et de la Motte, 2014). The small amount of data did not allow to draw conclusions but the evolution observed is hopeful because it shows the feasibility of the technique in the synovial fluid; this should be studied in the future.

Since knee OA-associated damages take place mostly within the synovial fluid, it makes sense to examine the MMPs proteolytic activity before and after CS injections to ensure that the drugs don't increase this activity (Heard et al, 2012).

Although approximatively 28 vertebrate MMPs have been identified according to their substrate preference, we chose to study gelatinases activity because it has been proved that MMP-2 and MMP-9 can serve as useful markers in OA study (Zeng et al, 2015).

their activity can be assessed by gelatin zymography, a technique which provides information about proteinases identities and a direct comparison of total potential activity which could contribute to (fibro)cartilaginous components breakdown (Volk et al, 2003; Carter et al, 2015).

MMP-2 and MMP-9 are secreted extracellularly by synovial cells and (fibro-) chondrocytes. MMP-2 seems to be only produced by resident cells while MMP-9 can be produced by invasive inflammatory cells in case of disease (Clegg et al, 1997). When secreted, the two MMPs can be activated and cleave growth factors, extracellular matrix, chemokines and cytokines, and that lead to activation of major signaling pathways involved in inflammation (Bauvois et al, 2012). Clegg et al. suggested that inactive MMP-9 form was soluble and free to diffuse from synovial fluid to the cartilage matrix and inversely (Clegg et al, 1997). The latent form of both MMPs are in greater concentration than the active form in healthy and inflamed joints (Trumble et al, 2001).

About their regulation, besides TIMPS regulation, MMP-2 is most probably regulated at the level of proenzyme activation, while MMP-9 appears to be regulated at a transcriptional level (Zrimsek et al, 2007; Heard et al, 2012).

Previous OA researches in dogs, horses and humans showed the almost absence of MMP-9 expression and constitutive MMP-2 expression in healthy joints (Mohtai et al, 1993; Trumble et al, 2001; Volk et al, 2003). A meta-analysis in man revealed a significant higher expression of MMP-2 and -9 in patients with OA than in the control group (Zeng et al, 2015). Animal studies showed similar results (Volk et al, 2003).

In vitro, Clegg et al. showed that methylprednisolone acetate and dexamethasone had a significant inhibitory effect on MMP-2 at high doses, but no effects on MMP-9. They also studied the effect of betamethasone but did not showed any significant effects neither on MMP-2 nor MMP-9 (Clegg et al, 1998).

To our knowledge, only two *in vivo* studies have assessed CS effects on MMP-9 activities in the synovial fluid. Shimizu et al. showed in man unchanged MMP-9 amounts five weeks after intra-articular injection of dexamethasone (Shimizu et al, 2010). A hypothesis is that it appears logical to have no inhibitory effects on MMP-9 in inflamed joints because CS injections deliver the drug locally and may act on resident cells but not on invasive inflammatory cells coming from blood vessels. However, like mentioned above, neutrophils granulocytes are the main source of synovial MMP-9 (Arican et al, 2000). Knych et al. found in exercised horses a notable increase in MMP-9 expression after triamcinolone acetonide injections. However, they found the same results in their control group and concluded that the increase could be due to repeated arthrocentesis (Knych et al, 2015).

Our study showed non-significant disruption of MMP-2 and MMP-9 activities in the synovial fluid after injection of triamcinolone hexacetonide. Trends were increases in MMPs activity after treatment. It could have been due for example to an increase in the activation of MMPs or to a decrease in the amount of available TIMPs. It could also have been due to a combination of these two hypothesis (Zrimsek et al, 2007).

However, even if the results would have been significant, we would not have been able to draw conclusions because linear regression models showed that age of animals had a significant impact on the MMPs activity. This significance, in contradiction with the preliminary study (though the results were close to significance), could be due to the small sample size or to the fact that the age of sheep are not exactly the same as in the first research population. Anyway, it would have make the results difficult to interpret if they had been significant.

VI. CONCLUSION AND PERSPECTIVES

The first part of this master thesis enabled to complement knowledge about the potential effects of ageing on menisci and synovial fluid in the ovine model. It was useful to know the naturally occurring evolution of some parameters (structure of menisci, HA in the synovial fluid, MMP-2 and -9 in the synovial fluid) to have baseline information in this research trying to study the effects of therapeutic agents. The preliminary study did not show significant impact of ageing. Nevertheless, as correlation between age and MMPs activities were close to the significance, we had to be careful during the analysis of CS effects on these parameters.

Conclusions of the second part of this master thesis are that: (i) 25mg of triamcinolone hexacetonide seem to have no significant effects on the biochemistry of menisci, i.e. on the water content, GAG content and collagen content after 1 month. (ii) The treatment response could be different between GAG contents of lateral and medial menisci. (iii) CS injections seem to have no significant effects on HA content and MMP-2 and -9 potential activities in the synovial fluid.

This study opens the door to new perspectives. As the effects of CS have been assessed in a healthy ovine model, the following step of the research could be to study CS effects in a clinical OA-induced ovine model. The disease could be induced by a meniscectomy.

In the future, the research project will continue by examining the CS effects on other biomarkers of the synovial fluid and (fibro) cartilaginous tissues. MMP-13 plays a central role in the cartilaginous degeneration associated with OA (Zhang et al, 2010). The small-peptide interleukin-1 (IL-1) is an inflammatory mediator which seems to increase the enzymatic activity in the synovial fluid and in the cartilage matrix of osteoarthritic joints (Chevalier et al, 1997). It will be therefore interesting to study the evolution of these biomarkers after CS treatment.

It will be useful to assess the effect of CS on the HA molecular weight more in depth. Electrophoresis gels and size exclusion chromatography will be interesting to study this subject and draw conclusions.

Techniques used in this master thesis could be refined. Firstly, to assess the GAG content, it would be more accurate to use detection methods which differentiate GAG subtypes. Chondroitin 6-sulphate (C6S) and chondroitin 4-sulphate (C4S) are the main GAG subtypes in the meniscal extra-cellular matrix (Herwig et al, 1984). It would be interesting to assess TH effects on the ratio C6S:C4S, as it has been done in the synovial fluid to study the effects of age and sex by Nakayama et al. (Nakayama et al, 2002). Secondly, to verify the HA dosage, besides the ELISA-like, another detection method could be used, such as the simple modified Alcyan blue technique introduced by Little et al. (Little et al, 1990). Finally, RT-qPCR analysis could be done again using a TissueLyser (Qiagen) to homogenize meniscal tissues, and editing primer melting temperatures.

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